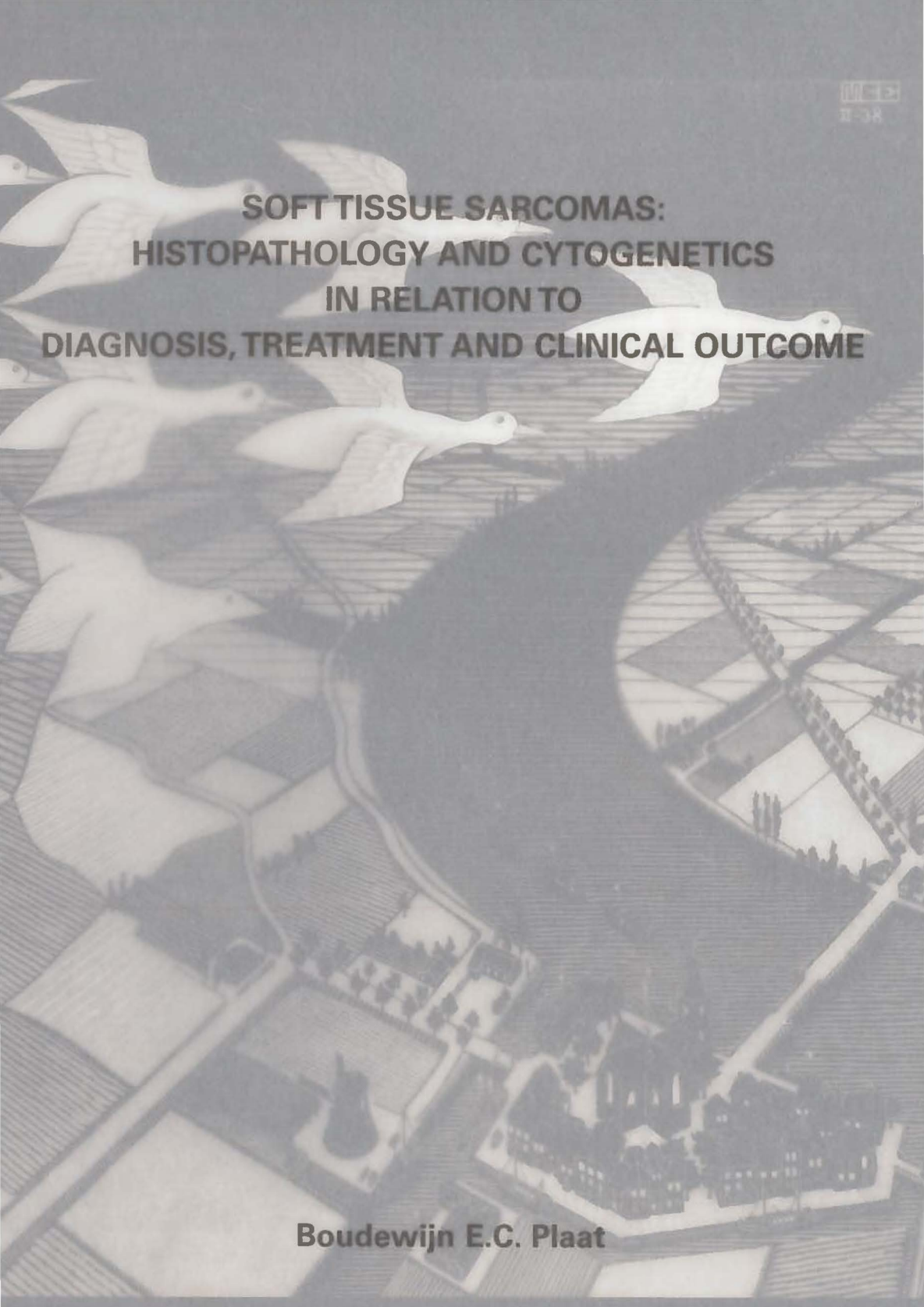

SOFT TISSUE SARCOMAS: HISTOPATHOLOGY AND CYTOGENETICS IN RELATION TO DIAGNOSIS, TREATMENT AND CLINICAL OUTCOME

Boudewijn E.C. Plaat

SOFT TISSUE SARCOMAS:
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STELLINGEN

behorend bij het proefschrift

Soft tissue sarcomas:
histopathology and cytogenetics
in relation to
diagnosis, treatment and clinical outcome.

van Boudewijn E. Ch. Plaat

15 december 1999

1. De tumorrespons van ledemaatperfusie met TNF-α en melphalan bij weke delen sarcomen wordt mede veroorzaakt door veranderingen in de celcyclus en in apoptose-mechanismen.

2. Hoewel multidrug resistance eiwitten in weke delen sarcomen frequent worden gedetecteerd, blijft de functie en de betekenis hiervan voor de kliniek onduidelijk.

3. Vele klinische kenmerken toegeschreven aan leiomyosarcomen berusten op een verkeerde diagnose.

4. Het gebruik van databases en computers in de analyse van chromosomale afwijkingen in solide tumoren, levert gemakkelijker interpreteerbare informatie op dan het gebruik van karyotypen alleen.

5. Het opzetten en gebruik van een database in klinisch gericht medisch wetenschappelijk onderzoek is geen randvoorwaarde maar een hoofdzaak.

6. Wanneer de kosten van het transport van materiaal op droogijs zouden worden berekend naar het gewicht van het pakket bij aankomst, zou dit stimulerend kunnen werken op de kwaliteit en snelheid van bezorging.

7. De invoering van de winkelwagentjesgulden heeft het sociale contact tijdens het winkelen in de supermarkt sterk bevorderd.

8. Volgens R. Pirsig is kwaliteit ondefinieerbaar, maar door het feit dat alle mensen het kunnen waarnemen is het gedefinieerd: het is menselijk.

9. Het steeds frequenter niet aangeven van richting in het verkeer, is een uiting van een toenemend gebrek aan identificatie aan de ander en dus een gevaar voor de medemens.

10. A little disorder can stimulate creativity enormously (naar: Captain Janeway, Star Trek Voyager).

11. Gokken wordt gezien als een zwakte van de mens, maar in het licht van de evolutie is het juist een sterkte.

12. Het gebruik van het woord "ritsen" langs de nederlandse snelwegen heeft taalkundig precies de tegenovergestelde betekenis van wat beoogd wordt.

13. Het toenemend gebruik van het woord "natuurlijk" is niet altijd even natuurlijk.

14. Beter méé verlegen, dan óm verlegen (dit proefschrift en alles wat er uiteindelijk niet in is gekomen).

RIJKSUNIVERSITEIT GRONINGEN

SOFT TISSUE SARCOMAS: HISTOPATHOLOGY AND CYTOGENETICS IN RELATION TO DIAGNOSIS, TREATMENT AND CLINICAL OUTCOME

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Paranimfen

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Christiaan C.W. Plaat

voor mijn vader en moeder

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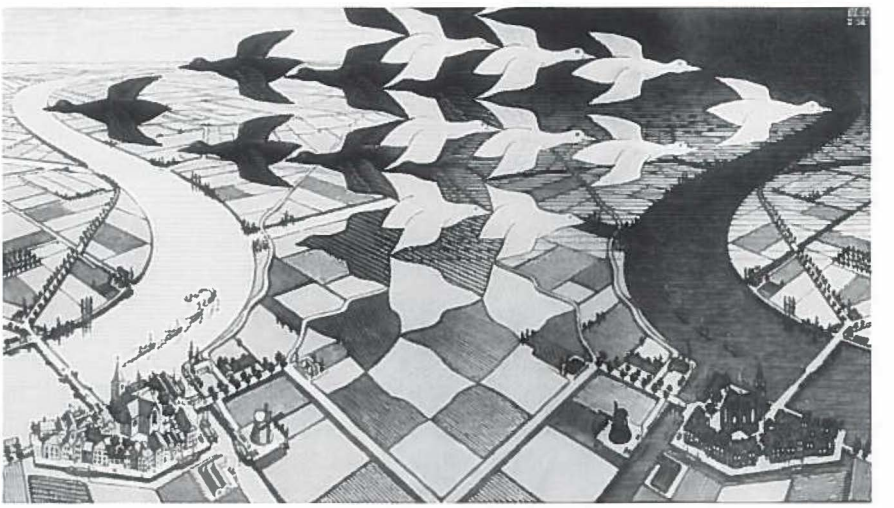

SOFT TISSUE SARCOMAS:

HISTOPATHOLOGY AND CYTOGENETICS IN RELATION TO DIAGNOSIS, TREATMENT AND CLINICAL OUTCOME

Cancer research has made tremendous progress during the last decades and many discoveries in the field of molecular biology have elucidated important mechanisms involved in cell biology and oncogenesis [1,2]. The development of new laboratory techniques, cancer cell lines and animal models has led to new insights in our understanding of oncogenesis, tumor progression and treatment effects. A new field of cancer research has been grown in which tumor biological parameters are translated into a clinical setting: the understanding of tumor biology is now intriguing the clinical world of oncologists [3].

Cancer cells defy normal controls on cell division and the apoptotic pathway. The processes leading to the irregular control are not only caused by successive mutations of the normal DNA, but subsequent tumor growth is influenced by various intracellular and extracellular processes as well. The net result is proliferative activity leading to tumor growth. Several authors have raised the question whether molecular markers and biological characteristics could predict treatment results and clinical outcome [4-7].

Non-skeletal malignant neoplasms originating from mesenchymal tissue, i.e. the soft tissue sarcomas (STS), are a rare group of cancers comprising approximately 1% of all malignancies [8,9]. The large proportion of the previous scientific studies that have analyzed STS have dealt with broad categories of different histological types or heterogeneous patients groups. As a consequence, data concerning single classes of STS or specific patients groups are limited. Factors contributing to the oncogenesis and the progression of STS and the prognosis of patients with STS are still poorly understood and tumor biological factors affecting prognosis of STS patients have not been studied widely.

Diagnosis of soft tissue sarcomas

Clinical evaluation

STS may occur anywhere in the body, but are most frequently found in the extremities and the retroperitoneum. STS can be found in both children and adults, although the distribution of the various histological types is different: (embryonal) rhabdomyosarcoma is a typical childhood STS whereas malignant fibrous histiocytoma is seldom observed in children. Most patients present with an asymptomatic mass as the only symptom. Physical examination, computed tomography (CT) or magnetic resonance imaging (MRI) are used to evaluate the extent of the local disease. In general, STS preferably metastasize to the lungs. Lymph node metastases are rarely found and are mostly associated with epithelioid sarcomas, rhabdomyosarcoma, synovial sarcomas and malignant fibrous histiocytoma. In the staging of STS, the staging system of the American Joint Committee on Cancer (AJCC) is commonly used. This staging system relies on histologic grade, tumor size, the presence of lymph node metastases and the presence of distant metastases, detected by physical examination, CT or MRI (Table 1).

For an adequate histopathological diagnosis, tumor tissue should be obtained by excisional biopsy (for small lesions) or incisional biopsy for lesions larger than 3 cm in diameter.

Table 1: The AJCC staging system

Stage	grade	tumor (∅)	Lymph node involvement	distant metastases
IA	I	< 5 cm	-	-
IB	I	≥ 5 cm	-	-
IIA	II	< 5 cm	-	-
IIB	II	≥ 5 cm	-	-
IIIA	III	< 5 cm	-	-
IIIB	III	≥ 5 cm	-	-
IVA	I-III	any size	+	-
IVB	I-III	any size	- or +	+

Imaging

Preoperative CT and MRI are important in the staging of STS [10]. With CT and MRI, not only primary STS, local recurrences and distant metastases can be detected, but also the size, the extension (CT), the relation with adjacent structures, can be determined. With CT and MRI, STS can be readily distinguished from the neurovascular structures without injection of contrast medium [8,11-14]. For the assessment of bone destruction or cortical erosian CT is preferred [8]. CT or MRI can further provide additional information regarding the presence of calcification, fat or blood within the tumor. However, CT or MRI cannot give an exact, reliable diagnosis nor can they reliably distinguish the several histologic types in STS or predict tumor grade. CT and MRI are further needed for the determination of a precise radiation therapy field.

Studies using radionuclides try to differentiate between benign and malignant soft tissue tumors. Thallium scans have shown not to be helpful for this purpose [15]. Imaging techniques such as positron emission tomography (PET) or single photon emission computerized tomography (SPECT), both able to visualize biologic processes, might be helpful in the assessment of tumor biology in STS and might provide an ancillary tool in the assessment of malignancy grade, tumor stage and treatment effects in STS [16,17]. Using PET with [^{18}F]-fluoro-2-deoxyglucose (FDG) it is possible to detect local recurrences of STS and to provide an indication of the malignancy grade of the recurrent lesion [18-22].

Histopathology

The pathogenesis of STS is not clear, although there might be a relation with previous injuries, irradiation, immunosuppression or lymphedema. Genetic factors might also contribute to the development of STS, since in up to 5% of the patients with neurofibromatosis-1, malignant peripheral nerve sheath tumors will develop [8] and in the Li-Fraumeni syndrome, a germline mutation of the p53 locus, also familial sarcomas have been found [23,24].

Classification - The heterogeneous group of STS can be classified in several histological types, depending on their histological features of differentiation as is shown in Table 2. Most common histological types in adult patients are malignant fibrous histiocytoma (MFH) (15%), liposarcoma (LPS) (15%), leiomyosarcoma (LMS) (10%), synovial sarcoma (10%), malignant peripheral nerve sheath tumor (MPNST) (10%), rhabdomyosarcoma (RMS) (10%), and sarcoma not otherwise specified (NOS) (10%).

Table 2: The primitive mesenchymal stem cell and the classification of soft tissue and bone tumors.

precursor	normal	benign (-oma)	borderline (-matosis)	malignant (-sarcoma)
fibroblast	fibrous tissue	fibroma	fibromatosis	fibrosarcoma
facultative histiocyte?	?	benign fibrous histiocytoma	dermatofibrosarcoma protuberans angiomatoid malignant fibrous histiocytoma	malignant fibrous histiocytoma
lipoblast	adipose tissue	lipoma	(lipoblastomatosis)	liposarcoma
rhabdomyoblast	skeletal muscle	(rhabdomyoma)	-	rhabdomyo-sarcoma
leiomyoblast	smooth muscle	leiomyoma	-	leiomyosarcoma
angioblast	blood and lymph vessel	(haem)angioma (lymph)angioma	angiomatosis	angiosarcoma
osteoblast	bone	osteoma	-	osteosarcoma
chondroblast	cartilage	chondroma	-	chondrosarcoma

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Immunohistochemistry has improved diagnosis of STS and its distinction from other tumor types such as carcinomas. For instance, the immunohistological detection of keratin is helpful in the differential diagnosis with carcinoma, whereas vimentin is predominantly found in malignancies arising from mesenchymal tissue. Although diagnosis of STS can be influenced by the subjectivity and experience of the individual pathologist, the classification of STS has further improved with the detection of differentiation specific antigens, i.e. in MPNSTs neural differentiation is associated with the expression of the S100 protein and in RMS muscle differentiation is associated with the expression of actin or desmin. Since then, the classification of

STS has changed and, as a consequence, the results of previous studies on STS have to be re-evaluated.

Tumor grade - The assessment of malignancy grade is of clinical and prognostic importance. There is discussion about the appropriate grading system for STS and several systems exist (Table 3) [25].

Table 3: histological parameters used in different grading systems

	Markhede [26]	Myhre Jensen [27]	Costa [28]	Coindre [29]
Cellularity	+	+	+	-
Differentation	-	-	-	+
Pleomorphism	+	+	+	-
Mitotic rate	+	+	+	+
Necrosis	-	+	+	+

From: Enzinger FA and Weiss SW [8]

In the commonly applied grading system according to Coindre *et al.*, also used in the studies described in this thesis, points are assigned to: differentiation level, necrosis and mitotic index (Table 4).

Table 4: The grading system modified from Coindre *et al.* [29]

parameter	condition	score
differentiation level		
	closely resembling normal tissue	1
	certain histogenetic classification	2
	undifferentiated*	3
necrosis**		
	no necrosis	0
	< 50%	1
	≥ 50%	2
mitotic count (/ 2 mm^2)		
	0 - 9	1
	10 - 19	2
	≥ 20	3
tumor grade		*total score:*
	I	2 , 3
	II	4 , 5
	III	6 , 7 , 8

**need for additonal immunohistochemistry*

***after macroscopic and microscopic assessment*

The counting of mitotic figures is one of the hallmarks in the assessment of malignancy grade in the various systems. However, the assessment of mitotic rate and the identification of mitotic figures can be difficult and has been shown to vary between observers [30-32]. Mitotic figures are not always easy to detect in haematoxylin-eosin stained sections, which are standard in the assessment of malignancy grade, and might even resemble apoptotic cells, the other side of the equilibrium [33]. Furthermore, the mitotic phase is a relatively short period in the cell cycle and proliferating cells are not only the cells in mitosis. Therefore, other methods have been introduced to examine the proliferative activity in malignant tumors.

Additional parameters:

Proliferation - One of the techniques to study proliferative activity is the detection of nuclei expressing the Ki-67 antigen, which is present in the nucleus of cells in all phases of the cell cycle except for the G0 and the early G1 phase. In a normal cell cycle, the cell will monitor its environment during the G1 (Gap 1) phase and, depending on intracellular and extracellular stimuli and the size of the cell, it will start to synthesize new DNA, whereas DNA replication itself happens during the S (synthesis) phase. After completion of the S-phase, DNA replication is checked during a safety phase or G2 (Gap 2) phase, before the cell will enter the most dramatic, but relatively short, phase in the cell cycle: mitosis (M-phase). In STS it has been found that high proliferation is associated with high malignancy grade [34,35].

Apoptosis - DNA damage will lead to cell cycle arrest with or without DNA-repair, depending on intracellular processes regulated by various genes and proteins i.e. p53, p21, p16, Rb, bcl-2, bcl-x, bax, the various cyclin dependent kinases and cyclins. Also extracellular signals can, via fas, fas-ligand and TNF-receptor-1, lead to the formation of a so-called death domain which will lead to formation of caspases and trigger the signaling cascade leading to apoptosis [36]. Apoptosis (so called "physiological"programmed cell death) is the final stage of the cell, which has activated a control mechanism leading to cleavage of the DNA and finally disposal of the cell. Cells undergoing apoptosis display cell shrinkage, loss of cell-cell contact, chromatin condensation and internucleosomal degradation of DNA [37]. Apoptosis can be visualized in paraffin embedded archival tissue by the TUNEL (Terminal deoxynucleotidyl Transferase (TdT) mediated dUTP Nick End Labeling) method. In STS it has been found that high malignancy grade is associated with low amounts of apoptosis [38], but a relation with histologic type has not been reported.

DNA-ploidy - In many malignant tumors an abnormal amount of DNA (DNA-aneuploid tumors) has been found. In many tumor types, DNA- aneuploid tumors, indicating gain or loss of DNA, have been associated with a more aggressive biological behavior than tumors with normal DNA content (diploid or euploid tumors). Evaluation of DNA-ploidy is a problem itself, since paraffin embedded tissue is often not interpretable and is depending on the preparation methods used [39]. Previous studies in soft tissue tumors have indicated that DNA ploidy is associated with malignancy and tumor grade, i.e. benign soft tissue tumors are almost always

diploid and sarcomas are either diploid or aneuploid [40]. It has been found that up to 66% of the STS is DNA aneuploid [41]. Aneuploidy is more common in high grade sarcomas than in low- or intermediate grade sarcomas [42]. Moreover, a positive correlation between DNA aneuploidy and high tumor grade or mitotic activity has been observed [35,41,43,44]. DNA-aneuploidy seems to be associated with specific histological types i.e., MFH and RMS are predominantly aneuploid [41,45]. The assessment of DNA-ploidy can be combined with cytogenetic analysis in order to obtain additional and more detailed information regarding abnormalities in chromosomal material [42].

Molecular (cyto)genetics - Cytogenetic analysis has proven to be an important tool in the detection of tumor specific alterations in the human genome [46-49]. This has been achieved by the karyotypic analysis of high numbers of tumors in which recurrent chromosomal alterations were found. These cytogenetic changes appeared to be related to the activation of an oncogene or the deletion of a functional tumor suppressor gene and further studies have finally resulted in the detection of genes which either may be characteristic for a tumor type or may be involved in cell proliferation in general [50-52]. Previous studies on cytogenetic abnormalities in sarcomas have focused on the support of the histological diagnosis [8,49,53]. Some studies have suggested that additional chromosomal changes occur in the process of tumor progression [54,55]. The findings of recurrent cytogenetic abnormalities in the various types of STS have lead to the discovery of certain fusion transcripts involved in the oncogenetic process of specific types of STS [56-59]. Certain translocations and their fusion transcripts are thought to be characteristic for specific STS and can be helpful in the diagnosis of STS (Table 5) [52,60,61].

Table 5: STS and their characteristic chromosomal translocations and fusion transcripts

Histopathology	cytogenetics	molecular genetics
myxoid and round cell liposarcoma	t(12;16)(q13;p11)	FUS-CHOP
synovial sarcomas	t(X;18)(p11;q11)	SYT-SSX1/SSX2
alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	FKHR-PAX3
	t(1;13)(p36;q14)	FKHR-PAX7
(extraskeletal) Ewing's sarcoma	t(11;22)(q24;q12)	EWS-FLI1
	t(21;22)(q21;q12)	EWS-ERG
Extraskeletal myxoid chondrosarcoma	t(9;22) (q22;q12)	EWS-TEC
Peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12)	EWS-FLI1
clear cell sarcoma	t(12;22)(q13;q12)	EWS-ATF1

The detection of the fusion transcripts is nowadays a relatively easy and quick method [62-69]. In this way, it is even possible to discriminate biphasic synovial sarcomas from monophasic synovial sarcomas with the detection of SYT-SSX1 and SYT-SSX2 fusion transcripts [70]. The detection of a t(11;22)(q24;q12) seemed to be characteristic for Ewing's sarcoma [52], but turned out to be involved in other

malignant mesenchymal lesions and other neoplasms, indicating a common oncogenetic pathway [71-74]. It has been reported that both EWS-FLI-1 and EWS-ERG fusion proteins may be responsible for the decreased ability of tumor cells to undergo apoptosis 7[5]. Furthermore, the same breakpoint 22q12 has also been observed in extraskeletal myxoid chondrosarcoma, but with other fusion partners [8,76].

A major problem in cytogenetic studies is the frequently observed complex karyotypes, in which non-random chromosomal abnormalities are difficult to detect. To discover meaningful chromosomal anomalies, which could be of diagnostic importance, karyotypes of relatively large number of tumors are needed as has been demonstrated by a recent study of Mertens et al. in various non-STS neoplasms [77].

Other molecular (cyto)genetic techniques such as comparative genomic hybridization (CGH), spectral karyotyping or multicolor fluorescence in situ hybridization might reveal additional information, especially in STS with difficulties in successful cytogenetic analyses, like MFH. In this way, CGH in MFH revealed that the most frequent gains were 1p31 (33%), and 9q31 (29%), whereas the minimal common regions of the most common losses were 13q21-22 (21%)[78].

Treatment of soft tissue sarcomas

Radical resection with wide surgical margins is the treatment of choice for local disease. In case of irresectable advanced extremity STS, hyperthermic isolated limb perfusion with tumor necrosis factor alfa (TNF-α) and melphalan (HILP-TM) with or without interferon gamma (IFN- γ)is used in several institutions with limb salvage in up to 80% of the patients [79-84]. Although the biological effects of HILP-TM are not completely understood, it is thought that TNF-α and/or IFN-γ induce apoptosis of vascular endothelial cells, while melphalan induces apoptosis of tumor cells [85].

In case of high grade STS or marginal surgery, postoperative radiotherapy is indicated [86]. Chemotherapy for primary tumors is applied in childhood RMS and in adult patients it should be considered when diffuse distant metastases have developed. In case of few solitary lung metastases, depending on the time lag after initial diagnosis, surgical resection can result in a prolonged (disease free) survival [86].

On a cellular level, both radiotherapy and chemotherapy intend to induce apoptosis and necrosis by damaging the DNA. Response to chemotherapy seems to be influenced by histological STS type i.e. leiomyosarcomas tend to respond poorly and liposarcomas relatively favorably to doxorubicin based chemotherapy [87]. Furthermore, high grade STS and STS with high S-phase fractions respond better to chemotherapy [87-89]. However, a study by Daugaard et al [90], in 94 STS treated with doxorubicin did not find a relation with either tumor grade or mitotic activity. The relation between the amount of apoptosis and responses to specific treatments has not been studied widely.

Multidrug resistance

Chemotherapeutic regimens do not always lead to tumor regression and tumor cells may appear to be resistant to chemotherapy. Overall, the response rates in metastatic adult STS patients to various chemotherapeutic regimens are between 20-40% [87]. Drug resistance can be caused by several mechanisms. The observed resistance to various structurally unrelated natural chemotherapeutic drugs has been called multidrug resistance (MDR). MDR is associated with the elevated expression of the MDR1 gene on 7q21 encoding P-glycoprotein (P-gp) and the multidrug resistance protein (MRP_1), the gene of which is located on 16p13. They both act as ATP dependent transmembrane pumps and are involved in the transport of cytotoxic agents towards the extracellular environment [91]. P-gp and MRP_1 have been shown to be clinically important in the chemotherapeutic responses in various malignancies [92-94]. Another protein which is associated with drug resistance in cancer is the lung resistance protein (LRP) which has been identified as the human major vault protein. The exact function of LRP, the gene of which is located on 16p11, is not completely understood. It is thought that it functions as a barrel shaped intracytoplasmic transporter which mediates nucleocytoplasmic and vesicular transport of cytotoxic agents [95]. LRP expression is of prognostic value in ovarian cancer and acute myeloid leukemia [96,97].

In sarcomas, P-gp expression might be associated with poor response to chemotherapy in Ewing's sarcomas [98] as well as with poor prognosis in osteosarcomas [99] and adult STS [100,101]. P-gp expression in childhood RMS was reported to be an extremely useful predictive factor of the response to chemotherapy, but the conclusions of this study of Chen et al. [102] were weakened by other studies [103]. Studies evaluating P-gp expression in adult STS vary considerably (18% to 62%) with regard to the amount of P-gp expression, which might be due to the methods used and the STS examined [100,101,104,105]. P-gp expression in synovial sarcomas seems not to be associated with gender, age, location, and proliferative activity of the tumors and was not influenced by chemotherapy, neither was response to chemotherapy related to P-gp expression [106]. The expression of the MDR protein P-gp has been associated with poor survival in a group of high grade STS of which a large number of cases received doxorubicin based chemotherapy [101]and its prognostic significance has been confirmed in other studies [100]. It was also found that P-gp expression is higher in high grade STS [100]. MRP_1 has been detected in Ewing's sarcoma [107] and co-expression of MRP_1 and P-gp was associated with tumor grade in STS [108]. MRP_1 has not been studied in relation to chemotherapeutic response or clinical outcome in sarcoma patients. LRP expression has hardly been studied in sarcomas.

Imaging of treatment response

CT and MRI are used in the post-therapy evaluation of treatment responses, the presence of local recurrences or distant metastases [8,10,109]. Other imaging techniques using the capacities of tumor biological parameters, have been studied for

their ability to determine responses to various form of treatment [16]. It was found that Thallium-201 scanning is valuable in the prediction of chemotherapy and radiotherapy responses [110-112]. PET using FDG is valuable in the assessment and prediction of treatment responses to HILP-TM [113,114], but can also be used in the assessment of responses to chemotherapy, as has been shown in other malignancies [19]. When comparing FDG-PET with 99mTc-MIBI SPECT with respect to diagnosis of recurrent or residual musculoskeletal sarcomas, FDG-PET showed higher sensitivity than MIBI-SPECT [115]. However, FDG-PET in the treatment evaluation can be complicated by the detection of a non-tumor specific signal, since a peripheral rim of FDG accumulation in the tumor was found in several studies, which correlated with the formation of a fibrous pseudocapsule or with the presence of inflammatory cells [113,116]. Shields et al. found that ^{11}C Thymidine-PET images show a decline in proliferative activity after successful chemotherapy and this technique seems superior to FDG-PET [117].

Clinical outcome of patients with soft tissue sarcomas

According to Enzinger and Weiss [8], prognosis of patients with STS is closely correlated with the ***histological type***: for instance patients with myxoid and well differentiated liposarcomas (LPS) have a far better prognosis than patients with malignant fibrous histiocytomas (MFH)[8]. Various univariate and multivariate analyses in clinicopathological studies on different STS types have concluded that age of the patient, the histological type, tumor grade, tumor size, tumor location and depth, the presence of distant metastases at presentation , and the adequacy of the surgical procedure is of important prognostic value [25,118-140]. Some STS, like myxoid LPS, are more associated with local recurrences than with distant metastases and some authors debate whether it is appropriate to include these tumors in studies evaluating prognostic factors [8,141]. Risk factors for ***local recurrences*** are: older age, local recurrence at presentation, positive surgical margins, histology of fibrosarcoma or malignant peripheral nerve sheath tumor [142]. Risk factors for ***distant recurrences*** are high tumor grade, tumor size, tumor depth, leiomyosarcoma and time to local recurrence [142-144].

The assessment of ***tumor grade*** appears to be the best predictor of prognosis. High ***proliferative activity*** as reflected by high mitotic counts [145-147]and high amount of Ki-67 expression [35,101,148-153] can predict poor survival. Furthermore, proliferative activity in STS seems to be associated with vascular invasion, metastasis and DNA-ploidy [34]. The prognostic significance of amount of apoptosis has not been established yet.

Studies evaluating ***DNA-ploidy*** in STS have revealed that non-diploid DNA content, alone or in combination with other prognosticators, correlates with poor outcomes [35,101,127,136,154-156]. Alvegard *et al.* found that DNA-aneuploid tumors had a worse prognosis than euploid tumors [156]. Some studies in heterogeneous groups of STS did not find a statistically significant relationship between DNA ploidy and

survival [126,157]. The significance of DNA ploidy as a prognostic factor, however, may differ among the various histological types of soft tissue sarcomas [126,145,158]. For example in synovial sarcomas a relationship was found between DNA ploidy and survival [159], whereas in liposarcomas DNA ploidy was not of prognostic value [160]. DNA aneuploidy is associated with higher metastatic risk [161], although the DNA-ploidy pattern in the metastatic lesions appears to be similar to that of the primary tumors [44].

The prognostic value of ***cytogenetics*** has not been established yet [56]. Grade III pleomorhphic STS have more chromosomal aberrations than grade II tumors, but no karyotypic pattern has been associated with clinical outcome or distant metastases [162]. In MFH 19p13 aberrations were related to a higher recurrence rate [163,164]. Other molecular cytogenetic techniques such as CGH might unveil prognostic information as has been shown in a recent study in with a correlation was found between gain of 7q32 and worse metastasis-free survival and overall survival of patients with MFH [78].

In conclusion, tumor biological factors affecting treatment response and prognosis of patients with STS have not been studied widely. Studies investigating the relation between tumor characteristics and clinical outcome may lead to future therapeutic improvements because they will enable us to predict tumor behavior on a more individual basis and allow more selective treatment strategies.

AIM OF THE PRESENT THESIS

The majority of the previous scientific studies within STS have dealt with heterogeneous groups of STS patients. As a consequence, data concerning specific patient groups and single classes of sarcomas are very limited. The aim of this thesis is to determine the relation of certain tumor biological parameters (i.e. proliferation, apoptosis, multidrug resistance, DNA-ploidy and chromosomal aberrations) with the histopathological diagnosis, treatment response and clinical outcome of patients with a STS.

Proliferation in neoplasms, as assessed by the counting of mitotic figures or the estimation of Ki-67 expressing cells detected by the MIB-1 antibody, has been shown to be of high prognostic value. The influence of professional experience with these methods could influence the assessment results. In **chapter 2**, these two methods will be compared in STS in a cross over design in which experienced observers are compared with less experienced observers.

Protein synthesis rate can be determined with L-[1-^{11}C]tyrosine positron emission tomography (TYR-PET). If a relation would exist between high protein synthesis measured *in vivo* and the amount of proliferative activity of STS, this could not only be useful in the assessment of malignancy grade *in vivo,* but also in the detection of those tumor regions with the highest proliferative activity. This would be helpful in the determination of the optimal localization for tumor biopsy or the

assessment of treatment response *in vivo*. In **chapter 3**, the relation between proliferation and protein synthesis measured with TYR-PET will be determined. Since PET needs a complex and expensive infrastructure, the relation between the more simple L-3-[Iodine-123]Iodo-alpha-methyl-tyrosine SPECT (IMT-SPECT) and the proliferative activity in STS will be studied for the same reasons **(chapter 4).**

Hyperthermic isolated limb perfusion with TNF-α and melphalan (HILP-TM) is used in patients with locally advanced soft tissue sarcomas. In **chapter 5**, the relation of tumor biological parameters (i.e. proliferative and apoptotic activity) with treatment response and clinical outcome of the HILP-TM treated patients will be established. Response to HILP-TM can be measured clinically by physical examination and CT or MRI. PET with either FDG or TYR reflects the metabolic properties of the tumor. The decrease in glucose metabolism or protein synthesis rate after HILP-TM has clinical importance if the HILP-TM induced changes in FDG-PET or TYR-PET would reflect changes in tumor proliferation. In **chapter 6**, the relation between proliferative activity before and after HILP-TM will be examined and FDG-PET will be compared with TYR-PET.

Treatment of metastatic or irresectable STS with chemotherapeutic agents is often complicated by poor treatment responses. Multi drug resistance (MDR) associated proteins like P-gp, MRP_1 and LRP could be responsible for the poor responses. In **chapter 7**, the distribution of P-gp, MRP_1 and LRP expression in a large series of STS will be determined in relation with histological type and tumor grade.

Recent studies indicated that leiomyosarcomas, and especially those with liver metastases, have a poor response to chemotherapeutic treatment. However, gastrointestinal stromal tumors (GIST) have been considered as LMS of the digestive tract for many years. In **chapter 8**, 29 deep seated LMS will be compared with 26 malignant GIST with regard to MDR proteins, clinical outcome and the development of liver metastases.

Patients with metastatic or irresectable STS are treated with polychemotherapy. In **chapter 9**, the P-gp, MRP_1 and LRP expression will be related to treatment response, overall survival and progression free survival in a group of patients with metastatic or irresectable STS treated with epirubicin, vindesine and ifosfamide.

Recurrent cytogenetic aberrations are of diagnostic value in STS. A major problem in cytogenetic studies is the difficulty of detecting non-random chromosomal abnormalities in complex karyotypes. To discover meaningful chromosomal anomalies, which could be of diagnostic or clinical importance, karyotypes of relatively large numbers of tumors have to be compared with other groups of tumors. In **chapter 10**, a computer assisted method will be introduced for the detection of non-random chromosomal aberrations in malignant peripheral nerve sheath tumors (MPNST). This method will be used for a direct comparison of sporadic vs. neurofibromatosis-1- associated malignant schwannomas.

The same method will be used in **chapter 11** to discover differences between mesenchymal gastrointestinal tumors ("GIST"), LMS and MPNST, since GIST have features of both smooth muscle or neural differentiation.
The importance of the t(11;22) in STS will be illustrated in **chapter 12** in which a t(11;22) in an extrarenal malignant rhabdoid tumor was found.

The 16p11 breakpoint, present in the t(12;16)(q13,p11) characteristic for myxoid liposarcomas, could affect the LRP-gene on 16p11.2. In **chapter 13** the expression of LRP in relation to the 16p11 breakpoint in liposarcomas will be described. To determine the expression of P-gp, LRP and MRP_1 in relation the cytogenetically detected breakpoints 7q21, 16p11 and 16p13, in a more heterogeneous group of STS, STS with 7q21, 16p11 or 16p13 breakpoints will be compared with STS with normal chromosomes 7 and 16 in **chapter 14**. The prognostic value of an abnormal karyotype in general with or without an abnormal DNA-content, will be examined in **chapter 15**.

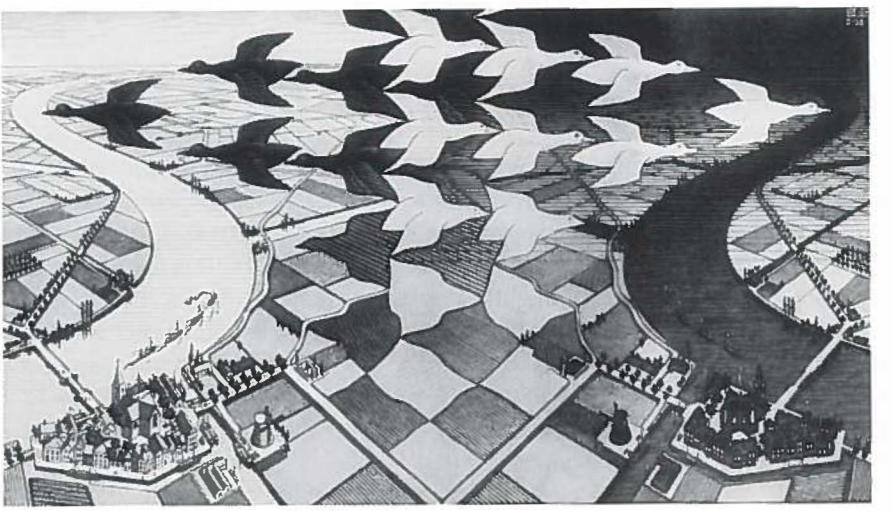
In **chapter 16** a summary of the findings will be presented and conclusions will be drawn.

PART I

HISTOPATHOLOGY

Chapter 2

OBSERVER RELIABILITY IN ASSESSMENT OF MITOTIC ACTIVITY AND MIB-1 DETERMINED PROLIFERATION RATE IN PEDIATRIC SARCOMAS



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SUMMARY

In H&E stained sections of 20 pediatric sarcomas the mitotic index was assessed by 4 experienced pathologists and 4 less experienced observers, without prior instructions. In adjacent sections immunolabeled for MIB-1, the proliferation index was assessed as the estimated percentage of labeled cells in the tumor cell population. Analysis of variance revealed that the variation between tumors explained 77% of the variation in mitotic indices in the group of experienced observers, as compared to 49% in the less experienced group. The variation between tumors explained 64% of the variation in proliferation indices in the experienced group and 71% in the less experienced group. The proliferation indices showed less variation between observers than the mitotic indices. No correlation was found between mitotic and proliferation indices. The results suggest that training is an important factor in the reliability of mitotic counting. The use of proliferation markers has a higher reproducibility, especially in less experienced observers. However, for clinical use it has the disadvantage of the more expensive and more time-consuming technique; moreover the biological significance of proliferation is as yet unestablished and may differ from that of mitotic activity.

INTRODUCTION

Microscopic evaluation of mitotic activity is a routine procedure in the assessment of the malignancy grade of tumors. This holds also for soft tissue sarcomas, in which different grading systems all include mitotic activity as a major parameter, which in turn appears to be related to clinical behavior [29,35,138,165]. It is therefore important to know the observer related reliability of such evaluations. In the last decade other methods to determine proliferative activity have been developed, among which MIB-1 labeling of proliferating cells is now commonly used. This method might appear more objective and easier to interpret than the counting of mitoses, but it is more labor intensive and costly. Moreover, it is uncertain whether mitotic activity and proliferation have the same biological significance.

Previous studies have focused on the reproducibility of mitotic counting with [31] or without [30] a strict protocol and training sessions or on the effect of delayed fixation [33]. In the current study the observer reliability in the counting of mitoses and the estimation of proliferation using MIB-1 labeling is assessed in a setting mimicking daily routine without previous training sessions. The same test set of 20 pediatric sarcomas was assessed by all observers, thus eliminating the effect of tissue procession. The correlation between both methods was tested. In addition, the influence of experience was evaluated by comparing groups of experienced and less experienced observers.

METHODS

Specimens of 20 pediatric sarcomas, i.e. 14 rhabdomyosarcomas, 2 epithelioid sarcomas , 2 synovial sarcomas, 1 fibrosarcoma and 1 malignant histiocytic tumor, were routinely processed and embedded in paraffin. In each case sections stained with H&E were used for mitotic counting; consecutive sections were labeled with MIB-1 (Immunotech S.A., Marseille, France) using alkaline phosphatase as chromogen and used for assessment of the proliferative activity.

From each pair of slides the same area (about 2 cm2) of viable tumor was indicated as target for analysis. For mitotic counting, each observer choose 2 mm^2 per slide in adjacent high power fields (400x magnification); since observers were using different microscopes each calculated the number of fields needed for this surface area. The recorded score was formed by adding the scores of each field. This approach was chosen to resemble daily practice, but it means that the observers were not necessarily scoring identical high power fields. Ten adjacent high power fields were also chosen by the observers for MIB analysis. MIB-scores were estimated in percentages of labeled tumor cells, an approach that has been found reliable [166]. The scores of each field were individually recorded, but for better comparison with the mitotic counts the total scores of 10 fields were added for the evaluation.

We compared 2 groups of observers, i.e. 4 experienced pathologists and 4 less experienced observers (a medical student, a PhD student, a pathology resident in the second year of training and a research technician). The unexperienced observers had a short introduction by the senior observer in the recognition of mitoses. Twenty specimens were scored by two different methods in a factorial experimental design. Slides were evaluated in 4 random orders, each order presented forward and backwards for counterbalancing reasons. The observers used their personal microscopes for evaluation. Scoring was spread over the day and not controlled for better resemblance of the evaluation with actual clinical practice. Data were gathered in a period of 14 days.

Data were entered in a D-base-4 database, exported to ASCII and transformed by PASCAL computer programs for entry in SYSTAT system files. Data and programs are available on demand.

RESULTS

The mitotic counts were after a log(1+x) transformation almost normally distributed (not shown). The MIB labeling indices were evaluated with the same transformation. These variance stabilizing transformations had a clear positive effect on the correlations and explained variance due to specimens in the analysis of variance.

Differences in means

We performed an analysis of variance to separate variance due to differences between observers and differences between specimens. In mitotic counting the

analysis of variance showed that the results are highly significantly different between observers as well as between specimens (table 1a). Seven percent of the variance was

Table 1: Analysis of variance in mitotic count.

a) for all observers.

SOURCE	SUM-OF-SQUARES		DF	MEAN-SQUARE	F-RATIO	P-value
Observer	7%	10.340	7	1.477	4.034	<0.001
Specimen	59%	84.223	19	4.433	12.106	<0.001
ERROR	34%	48.699	133	0.366		

N: 160; multiple r: 0.812; squared multiple r: 0.660.

b) for experienced observers.

SOURCE	SUM-OF-SQUARES		DF	MEAN-SQUARE	F-RATIO	P-value
Observer	7%	6.707	3	2.236	8.505	<0.001
Specimen	76%	71.092	19	3.742	14.234	<0.001
ERROR	16%	14.984	57	0.263		

N:80; multiple r: 0.916; squared multiple r: 0.839.

c) for less experienced observers.

SOURCE	SUM-OF-SQUARES		DF	MEAN-SQUARE	F-RATIO	P-value
Observer	4%	1.968	3	0.656	1.635	0.191
Specimen	50%	23.972	19	1.262	3.144	<0.001
ERROR	47%	22.875	57	0.401		

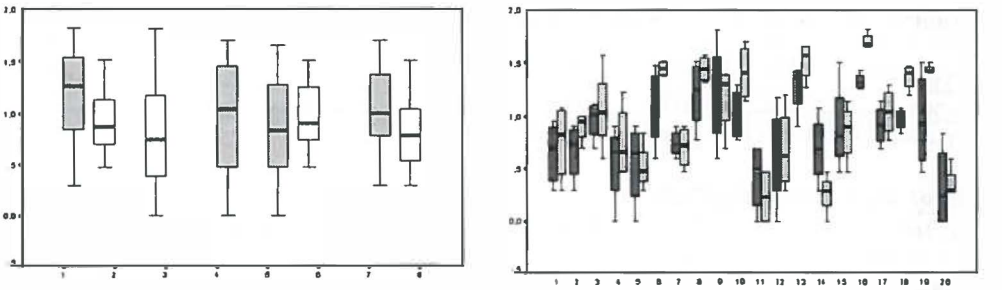
N: 80; multiple r: 0.729; squared multiple r: 0.531.

Dependent variable: log(mitosis+1); DF: degree of freedom (=n-1); sum of squares: relative proportion in total variance; mean square: relative proportion in variance divided by degrees of freedom.

related to differences between observers, 59 % was related to differences between specimens and 34 % was unexplained (random error and interactions). The experienced observers counted more mitoses than the less experienced observers, i.e. mean values of 16/2mm^2 versus 11, respectively (fig. 1). Separate analyses of variance for the experienced and the less experienced observers (tables 1b and 1c), showed that the experienced observers had highly significant differences in mean levels, but the specimens accounted for most of the variance. The less experienced observers did not differ significantly in mean levels assigned, but the error variance was much higher.

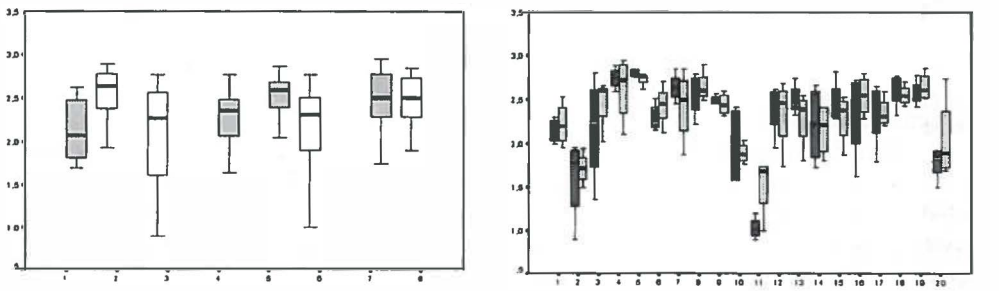
In the estimation of MIB labeling index the analysis of variance showed that the results are highly significantly different between observers and between specimens (table 2a). Eleven percent of the variance was related to differences between observers, 64 % was related to differences between specimens and 25 % was unexplained. The experienced and the less experienced observers had comparable scores, i.e. means of 29 and 30%, respectively (fig.2). The less experienced observers had the same significant

influence on the results, but they had an even lower error variance (18%) than the experienced observers (26%) (tables 2b and 2c).



A. **B.**

Figure 1
Mitotic index: (A) boxplot for variance in each observer for all sections (experienced: gray; less experienced: white) and (B) boxplot for variance per section for all observers (experienced: light gray; less experienced: dark gray)



A. **B.**

Figure 2
Proliferation: (A) boxplot for variance in each observer for all sections (experienced: gray; less experienced: white) and (B) boxplot for variance per section for all observers (experienced: light gray; less experienced: dark gray)

Table 2: Analysis of variance in MIB-1 labeling index.

a) for all observers

SOURCE	SUM-OF-SQUARES		DF	MEAN-SQUARE	F-RATIO	P-value
Observer	11%	18.553	7	2.650	8.668	<0.001
Specimen	63%	103.984	19	5.473	17.898	<0.001
ERROR	25%	40.669	133	0.306		

N:160; multiple r: 0.866; squared multiple r: 0.751.

b) for experienced observers.

SOURCE	SUM-OF-SQUARES		DF	MEAN-SQUARE	F-RATIO	P-value
Observer	11%	7.166	3	2.389	8.054	<0.001
Specimen	63%	40.180	19	2.115	7.131	<0.001
ERROR	26%	16.905	57	0.297		

N:80;multipler:0.858;squaredmultipler:0.737.

c)for less experienced observers.

SOURCE	SUM-OF-SQUARES		DF	MEAN-SQUARE	F-RATIO	P-value
Observer	11%	11.247 3	3	0.749	12.047	<0.001
Specimen	71%	69.829	19	3.675	11.810	<0.001
ERROR	18%	17.739	57	0.311		

N:80;multipler:0.906;squaredmultipler:0.820.

Dependent variable: log(MIB+1). DF:degree of freedom (=n-1); sum of squares: relative proportion in total variance; mean square: relative proportion in variance divided by degrees of freedom.

Correlation analysis

In order to compare the scores of the individual observers with the mean score of all observers, a principal component analysis was performed. With this method a perfect coincidence would result in a loading factor of 1 for the principal component. The analysis revealed for the mitotic counting that the experienced observers had high factor loadings on the first principal component, i.e. 0.91, 0.80, 0.94 and 0.95, respectively, while the less experienced observers had much lower factor loadings of 0.76, 0.76, 0.55 and 0.67, respectively. The first principal component accounted for 64% of the total variance.

For the MIB labeling index the results did not differentiate between experienced and less experienced observers. The factor loadings for the experienced observers were 0.95, 0.94, 0.99 and 0.96, respectively, and for the less experienced observers 0.97, 0.91, 0.96 and 0.95, respectively. For the MIB labeling index all observations of the assessed fields per slide were used in a principal components analysis. The first principal component has been computed for each observer on the basis of all measurements. This factor explained for the experienced observers 88.9, 81.8, 64.3 and 75.4 %, respectively, of the total variance. For the less experienced observers the explained variance for the first principal component was 86.6, 72.1, 79.6 and 84.2%, respectively. This implies that

the consistency between experienced and less experienced observers does not differ very much. The first principal component accounted for 91 % of the total variance.

Correlation between mitotic count and MIB labeling index

A principal components analysis revealed that there were two important orthogonal principal components. The first one was the MIB score (0.9x MIB-0.25x mitoses) and the second one was a sum of the mitotic count (for the largest part) and the MIB score (0.8x mitoses + 0.25x MIB). The variance accounted for by these two components was 48.8% by the first and 30.5% by the second. Finding an important second component indicates that the two methods measure different parameters.

DISCUSSION

The current study determined the observer reliability in mitotic counting and in the estimation of MIB labeling index in a series of pediatric sarcomas. It further studied the influence of experience in both assessments and compared the two techniques with each other. The study design was such that it resembled daily practice as much as possible. In particular, the observers used their own microscopes and archival material was used, thus reflecting tissue processed at different time points and different fixation periods. The reliability of observations has to be evaluated relative to the variance in the observed material: if all specimens have about the same mitotic count, the correlation between observers will necessarily be low, if the variance is extreme, the correlation between observers will be very high [167]. In the current investigation no selection was made such that the specimens would represent the full spectrum, which makes it easier to evaluate the meaning of differences between observers for actual clinical scoring. In the study by Donhuijsen et al. [30] very extreme slides were used, but significant differences between observers were found as well. Differences between observers exist as a difference in mean, lack of (linear) correlation and in variance. Differences in means are analyzed by analysis of variance. The purpose of the principal components analysis is to evaluate the correlation between and within observers and methods. The main results for both mitotic counting and MIB labeling index estimation are that there are highly significant differences between observers in mean levels. The interpretation of these differences is difficult. It is conceivable that the experimental setup introduced an important source of variation between observers: observers were free to choose their high power fields in a designated area. Some may have chosen more variable fields as e.g. the observer with the lowest common first component in the principal components analysis, while other observers may have chosen more similar fields. It is impossible to evaluate this difference in terms of worse or better, because there is no golden standard. It indicates however, that there is a problem, where standardization of procedures, e.g. field selection could assist in reducing unwanted variation between observers. Possible solutions can be found in systematic training, where observers are trained in consistent selection of observation fields, as well as in training to discriminate mitotic cells more consistently [33]. An improvement in consistency by bringing the 'personal bias' of

observers closer together is in principle possible. One approach would be to measure the individual bias and to correct for this bias. This can be done by giving percentage scores related to results on a standard sample, that indicates for each observer in which percentile an observation falls. Such a procedure would also have the same effect as taking the log of observations, which gives a score that is better interpretable than the raw score, as the variance due to observers decreases and the variance due to specimens increases after this transformation.

Comparison between experienced and less experienced observers revealed consistent differences with respect to the evaluation of mitotic counting. Less experienced observers had a lower correlation with the average score, as follows from the lower factor loadings on the first principal component. This implies that experience is evidently required to be able to recognize a mitosis, as previously reported [30]. The data further indicate that MIB labeling index can be consistently scored by both experienced and less experienced observers. This suggests that this technique is simpler to score reliably, but since MIB labeling is not (yet) a routinely applied technique the difference in experience between the two groups in this respect is smaller than for mitotic counting.

Mitotic counting and MIB labeling are both supposed to assess cell growth. A positive correlation between the two might therefore be expected and was indeed found in two earlier studies in soft tissue sarcomas [150,168], but not in another [35]. In the current analysis two relatively independent principle components were established, i.e. the MIB-1 score and the sum of the MIB-1 score and mitotic count. These differences between the various studies may be due to different techniques, e.g. in the study by Jensen et al. [168] the comparison was made between the counts of MIB labeled cells and classes of mitotic indices, whereas in the current study the MIB labeling index was estimated and compared to the full spectrum of mitotic activity. Moreover, our analysis was performed after log-transformation of the scores. The absence of a correlation may also indicate that mitotic activity and MIB measure different phenomena, e.g. related to the variation in fraction of the cell cycle that is taken up by the mitoses or the number of proliferating cells that actually goes in mitosis. Finally, these parameters may differ between tumor types as demonstrated in a recent study in carcinomas [169].

In conclusion, the current study indicates that mitotic counting is reliable when done by experienced observers. It further shows that some important aspects of inter-individual scoring can be analyzed in a simple experimental design. The results suggest that there is room for improvement and set at the same time a minimum for the magnitude of correlations between observers. The MIB measurement has the advantage that it can be scored by relatively inexperienced personnel. However, it is more laborsome and costly. Moreover, the clinical relevance of mitotic count and MIB labeling index has to be evaluated, because they may measure different aspects of a specimen.

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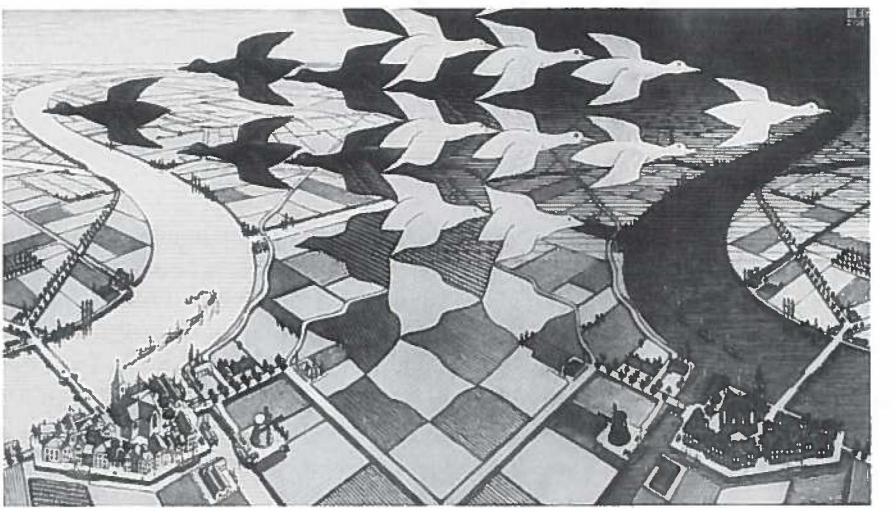
Chapter 3

PROTEIN SYNTHESIS RATE MEASURED WITH L-[1-^{11}C]TYROSINE POSITRON EMISSION TOMOGRAPHY CORRELATES WITH MITOTIC ACTIVITY AND MIB-1 ANTIBODY-DETECTED PROLIFERATION IN HUMAN SOFT TISSUE SARCOMAS



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SUMMARY

Protein synthesis rate (PSR) can be assessed in vivo using positron emission tomography with L-[1-^{11}C]tyrosine (TYR-PET). Biological activity of soft tissue sarcomas (STS) can be measured in vitro by mitotic rate and amount of proliferating cells. In STS the grade of malignancy, in which mitotic index plays a major role, is considered to be the major standard in predicting biological tumour behaviour. This study was designed to test the validity of TYR-PET in relation to different histopathological features. In 21 patients with untreated STS, the PSR was measured using TYR-PET. The number of mitoses was counted and tumours were graded according to the grading system of Coindre et al. (Cancer 1986; 58:306-309). Proliferative activity was assessed by immunohistological detection of the Ki-67 nuclear antigen using MIB-1 monoclonal antibody. To test the association between the PSR and these tumour parameters, a correlation analysis was performed. A significant ($p<0.05$) correlation was found between PSR and the Ki-67 proliferation index ($r=0.54$), and between PSR and mitotic rate ($r=0.64$). There was no correlation between PSR and tumour grade. The present study in malignant soft tissue tumours connects in vivo tumour metabolism as established with TYR-PET to tumour activity measured in vitro and indicates that the non-invasive method of TYR-PET can estimate the mitotic and proliferative activity in STS.

INTRODUCTION

Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumours arising from tissue of mesenchymal origin and account for approximately 1% of all malignancies. Patients with STS usually have a poor prognosis. The most significant prognostic factor in STS is tumour grade, i.e. a higher tumour grade is associated with a worse prognosis [8,128,143]. Tumour grade is determined by mitotic activity, amount of necrosis and differentiation grade. Also the number of proliferating cells has been associated with the grade of malignancy [34]. Proliferation can be assessed by the detection of Ki-67, which is a nuclear antigen present in all parts of the cell cycle except for the G-zero phase of the cell cycle (G0), in which the cells are withdrawn from the cell cycle, and the early Gap 1 phase (G1), the phase of the cell cycle before the start of DNA synthesis [170].

Tumour metabolism can be determined *in vivo* by positron emission tomography (PET), in which radiopharmaceuticals labelled with positron-emitting nuclides are used to assess metabolic processes. A relation was found between glucose consumption as measured with 2-[^{18}F]-fluoro-2-deoxyglucose (FDG)-PET and tumour grade in groups of patients with various benign and malignant musculoskeletal tumours [21,171]. Because with FDG-PET one cannot discriminate between (malignant) viable tumour and inflammatory reaction [113], the radiopharmaceutical L-[1-^{11}C]tyrosine (TYR) is currently under investigation for its use in the detection and therapy evaluation of soft tissue tumours at the Groningen

University Hospital. With TYR-PET several types of tumours including STS can be visualised and the protein synthesis rate (PSR) can be quantified [172-176]. The current study in malignant soft tissue tumours investigated the correlation between *in vivo* tumour metabolism using TYR-PET and *in vitro* biological activity as reflected by tumour grade, number of mitoses and amount of proliferating cells.

MATERIALS AND METHODS

Patients

TYR-PET was performed in twenty-one patients, 14 males and 7 females, with a histological diagnosis of a malignant mesenchymal tumour that measured at least 2 cm in diameter and was located in the soft tissues. The tumours were originally detected by magnetic resonance imaging or by computed tomography. Mean age at the time of diagnosis was 56 (range 24 - 83) years. Eighteen tumours were located in the lower limb (85%), one in the upper limb (5%), one in the gluteal region (5%) and one in the upper part of the back (5%).

The TYR-PET study for patients with locally advanced STS was approved of by the medical ethics committee of the Groningen University Hospital and all patients gave informed consent.

Methods

In all patients tumour tissue was obtained by diagnostic incisional biopsy within 8 weeks (mean: 16 days; range: 1-55) after PET and the histological diagnosis was made on haematoxilin-eosin stained paraffin sections with or without additional immunohistological stains. All tumours were classified according to Enzinger and Weiss into 10 different histological types [8]. The STS were graded according to the grading system of Coindre *et al.* in which points are assigned to differentiation level, mitotic index and necrosis [29]. This resulted in 6 grade I (29%), 4 grade II (19%) and 11 grade III (52%) STS (Table 1). The number of mitotic figures per 2 mm^2 was counted in fifteen adjacent fields on haematoxilin-eosin stained paraffin sections.

Proliferating cells were detected using the monoclonal antibody MIB-1 (Immunotech S.A., Marseille, France), which recognises an epitope of the Ki-67 antigen. Immunohistochemistry was performed on paraffin sections (4 μm) according to a method modified from Shi *et al.* [177,178]. In short: after heating on a hot plate, the slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After two times heating in an autoclave for 10 minutes at 110° C in cooking solution pH=6.0, the slides were incubated with a 1:400 dilution of the antibody in bovine serum albumin pH=7.4. The primary antibody was detected with a biotinylated secondary antibody (multilink) followed by a streptavidine-alkaline phosphatase conjugate (Ready-to-Use Link and Label, Biogenex, San Ramon CA, USA). Final colour was developed using bromochloroindolyl-phosphate 4-nitroblue-tetrazoliumchloride (BCIP-NBT; Boehringer, Mannheim, Germany).

For measuring the Ki-67 labelling index (LI) we used ocular micrometry on a Leica microscope (Rijswijk, the Netherlands) by using an eyepiece grid at x400 magnification. Fifteen fields were randomly selected throughout histologically viable areas. Endothelial cells, inflammatory cells and necrosis were excluded. The number of positive nuclei was then divided by the total number of nuclei in each of the fifteen fields to calculate an index per field. The Ki-67 LI representing proliferative activity was defined as the mean of the indices of the fifteen fields.

Table 1. Patient characteristics, histology and tumour grade.

Patient	Sex	Age	Diagnosis	Tumour grade
1	M	48	fibrosarcoma	I
2	M	49	malignant hemangiopericytoma	I
3	M	58	myxoid liposarcoma	I
4	M	37	myxoid liposarcoma	I
5	M	68	well differentiated liposarcoma	I
6	M	42	sarcoma not otherwise specified	I
7	M	66	malignant fibrous histiocytoma	II
8	F	69	malignant fibrous histiocytoma	II
9	M	48	myxoid liposarcoma	II
10	M	24	synovial sarcoma	II
11	F	69	fibrosarcoma	III
12	M	45	leiomyosarcoma	III
13	M	66	malignant fibrous histiocytoma	III
14	F	67	malignant fibrous histiocytoma	III
15	F	83	malignant fibrous histiocytoma	III
16	F	32	malignant Schwannoma	III
17	M	67	pleiomorphic liposarcoma	III
18	M	74	sarcoma not otherwise specified	III
19	M	74	sarcoma not otherwise specified	III
20	F	69	sarcoma not otherwise specified	III
21	F	25	synovial sarcoma	III

PET experiments.

Patients fasted for at least eight hours before the study, but could drink water and take their usual medication. A venous cannula was placed in the antecubital vein of one of the forearms to allow easy injection of TYR. Another cannula was placed in the radial artery of the contralateral arm to assess the arterial input function and metabolites. The patient was positioned in the PET-scanner (ECAT 951/31, Siemens/CTI, Knoxville, TN, USA) with the tumour in the field of view. After a 20 minute transmission scan to correct for attenuation, 370 MBq TYR was injected via the venous access as a 1 minute bolus. Data acquisition started at the time of injection (time frames 10x30, 3x300, 3x600 seconds). During the study arterial blood samples

were taken for the assessment of the plasma tyrosine activity curve and of its radiolabelled metabolites ($^{11}CO_2$ and ^{11}C-labelled proteins). The results from these measurements were similar as in previous studies [172]. PET images were displayed in transaxial projections on a computer display applying standard ECAT software (Siemens/CTI, Knoxville, TN, USA). Tumour definition was done by visual inspection based on computed tomography or magnetic resonance images. The activity in the selected pixels was averaged and the corresponding time-activity curve was calculated. Combining this averaged time-activity data with the plasma input data (corrected for $^{11}CO_2$ and ^{11}C-proteins), the average PSR is calculated in nmol/100g tumour tissue/minute using a compartmental model describing the kinetics of L-[1-^{11}C]tyrosine as described previously [172]. In brief, it models the plasma-tissue transport of tyrosine and its incorporation into protein. In addition, tissue metabolites ($^{11}CO_2$) and their transport to plasma are included also. The radiochemical and enantiomeric purity were both >99% and the specific radioactivity was >18.000 Bq/mmol [172]. Both the maximal measured PSR (PSRmax) from the ten pixels with maximum activity and the average PSR for the whole tumour (PSRavg) were calculated.

Statistical analysis

Since the distribution of all tumour parameters except for tumour grade was normal, a two-tailed Pearson's correlation analysis was performed to quantitate the degree of correlation. To determine the relation between PSR and tumour grade a Spearman's rank correlation coefficient was calculated. A p-value of < 0.05 was considered to be significant. Statistical analysis was performed using Graphpad Prism 2.0 (Graphpad Software Inc., San Diego, California, USA).

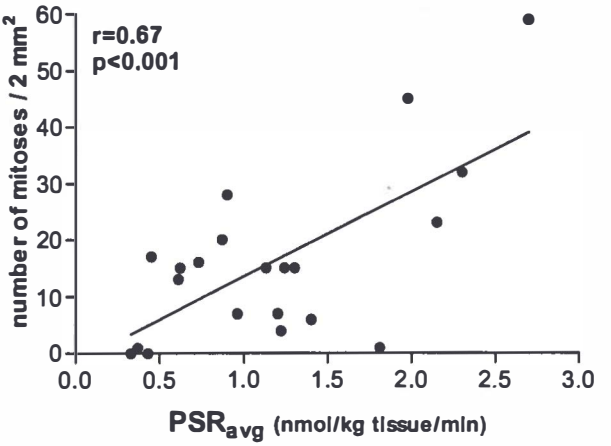
RESULTS

The PSRavg was 1.18 ± 0.68 nmol/kg tissue/min (mean ± SD) and ranged from 0.33 to 2.70 nmol/kg tissue/min, whereas the PSRmax was 2.46 ± 1.45 (range 0.64 - 5.66). In the patient with the lowest PSRavg (0.33 nmol/kg tissue/min) it was not possible to calculate the PSRmax. A high correlation was observed between PSRavg and PSRmax ($r=0.95$, $p<0.0001$).

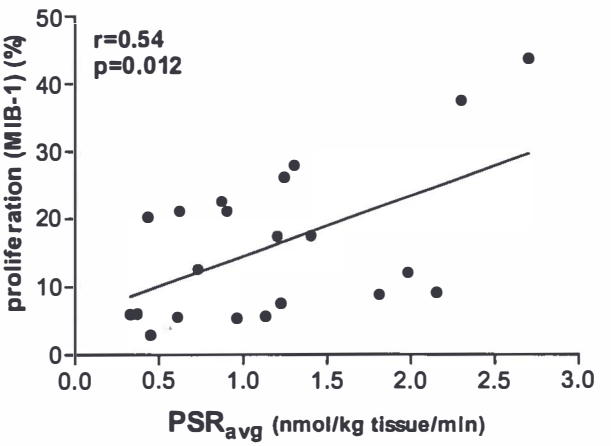
No correlation was found between *tumour grade* and either of the PSRs (Table 2). The PSRavg was 1.02 ± 0.58 for the 6 grade I STS, 0.89 ± 0.43 for the 4 grade II STS and 1.37 ± 0.77 for the 11 grade III STS. The mean PSRmax was 2.40 ± 1.31 for the 5 grade I STS, 1.53 ± 1.01 for the 4 grade II STS and 2.84 ± 1.58 for the 11 grade III STS.

A significant correlation was found between the *mitotic activity* and PSRmax as well as PSRavg (Table 2; Fig. 1*A*). Mean mitotic rate was 16 ± 15 per 2mm^2 (range 0 - 59 /2mm^2).

The amount of *proliferation* correlated with PSRmax and PSRavg (Table 2; Fig. 1*B*). Mean proliferation was 16 ± 11% (range 3 - 44%). Two examples of TYR-PET images with corresponding MIB-1 stained sections are shown in Fig. 2.



A.



B.

Figure 1
Scatter plots showing the relation between **A.** the PSRavg and number of mitoses per 2 mm² and **B.** the PSRavg and MIB-1 labelling index.

DISCUSSION

Mitotic activity and amount of cell proliferation in a tumour are important determinants of tumour malignancy and are associated with prognosis i.e. high numbers of dividing and proliferating cells are related with a worse prognosis in many malignancies [8,34,35,146,150,179-183]. High proliferative activity in a tumour indicates that a high proportion of tumour cells has entered the cell division cycle, whereas a

Table 2. Correlation coefficient and two-tailed p-value of the studied histological parameters and both PSRavg and PSRmax.

		tumour grade	mitoses/2mm^2	proliferation
PSRavg	r	0.22	0.67	0.54
	p	NS[a]	<0.001	<0.05
PSRmax	r	0.14	0.64	0.54
	p	NS[a]	<0.01	<0.05

[a] NS: p>0.05

high mitotic activity implies that a large number of cells is in the final phase of the cell cycle. The total protein content of growing cells increases continuously throughout the cell cycle [2]. Tyrosine is used in the production of various proteins and the PSR, as measured by TYR-PET, represents the biological activity of the viable parts of a tumour [172]. Malignant tumours with high numbers of proliferating cells are expected to have a relatively high protein metabolism and can therefore be detected by TYR-PET. This study indeed demonstrates the correlation between PSR and both mitotic and proliferative activity in malignant soft tissue tumours. The observed link between PSR and these indicators of prognosis stress the potential role of TYR-PET in predicting clinical outcome and indicate that the specific parts of STS with relatively high mitotic and proliferative activity can be discovered *in vivo*. It would be interesting to know if a stronger correlation could be detected by taking larger samples to quantitate the numbers of mitoses or proliferating cells. Moreover, in this study the tumour samples were randomly taken and not focused on the areas with the highest PET signal. In view of the correlation between the PSR and the markers of proliferation, the correlation coefficients are expected to increase when biopsies are taken from areas with the highest PET signal. PET directed incisional biopsies may also be helpful to avoid large necrotic parts of the tumour and make histological diagnosis and assessment of the malignancy grade more accurate.

In this study tumour grade was not found to correlate with PSR as measured in the viable parts of the tumours. This might be due to the fact that the amount of necrosis, in which no protein synthesis is present, is part of the used grading system of Coindre *et al.* [29]. A large amount of necrosis, indicating a high tumour grade, decreases the PSR because the non viable parts of the tumour have no protein synthesis and the diffuse small necrotic foci in a tumour cannot be visualised by TYR-PET but are incorporated in the microscopic grading.

Previous studies used FDG-PET in the analysis of soft tissue tumours and a relation was found between tumour grade and FDG uptake 21,171,184,[185]. However, the results of some of these studies cannot be directly translated to STS since the authors combined benign and malignant soft tumours to assess the relation between tumour grade and FDG-PET or included different types of malignant tumours and did not use the appropriate grading system for these tumours. The present study was confined to a homogeneous group of STS which were uniformly graded.

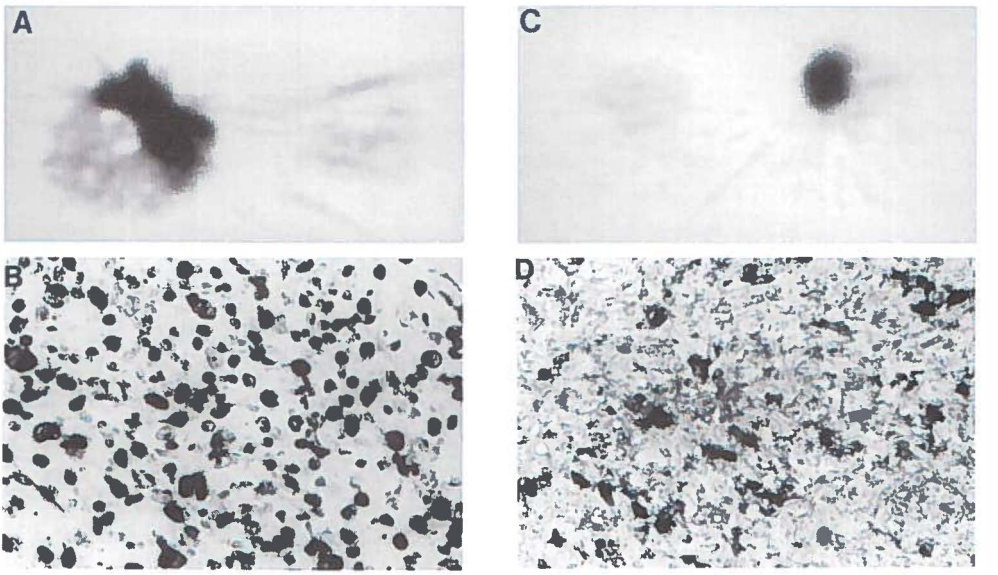


Figure 2
A. TYR-PET image of a sarcoma not otherwise specified with a relatively high PSR and **B.** corresponding MIB-1 stained tissue section (400x) of the tumour showing a high amount of proliferating cells. All dark nuclei express Ki-67 and have entered the cell cycle. **C.** TYR-PET image of a malignant Schwannoma with a relatively low PSR and **D.** corresponding MIB-1 stained tissue section (400x) showing a low amount of proliferating cells.

Chemotherapeutic and radiotherapeutic treatment responses are associated with reduced mitotic activity and proliferation in various cancers [186-188]. The observed correlation between these tumour parameters and the PSR implies that TYR-PET could be used in evaluating treatment responses *in vivo*.

In conclusion, this study established the correlation between *in vivo* tumour metabolism and the *in vitro* biological activity of STS and validates TYR-PET as an important tool in the *in vivo* evaluation of malignant soft tissue tumours. Further studies overcoming the limitations of the present study are warranted. They have to be performed in a larger group of patients using tumour material obtained after PET directed biopsies, with a minimum period between biopsy and PET. If large excision specimens could be examined, it is advised to study tumour proliferation and other cell cycle parameters as well as amount of cell death in areas of both high and low PET signal within the same tumour.

Acknowledgements
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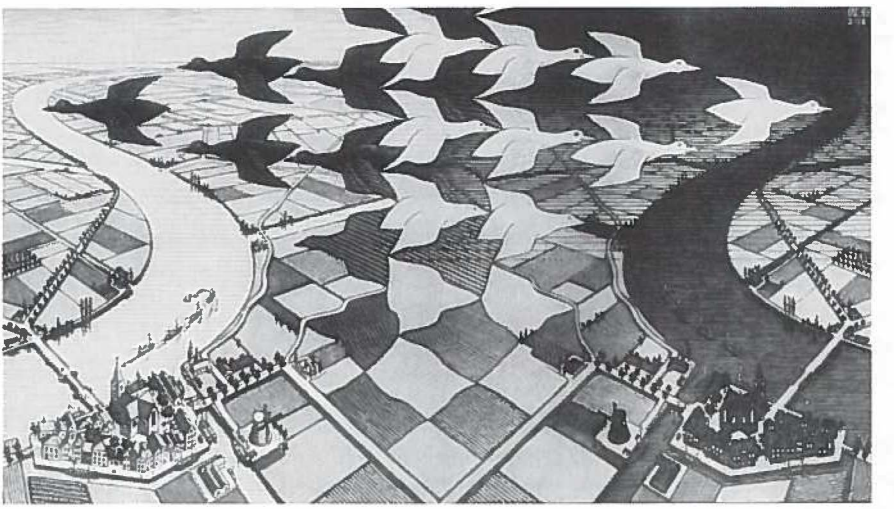
Chapter 4

IMAGING OF SOFT-TISSUE TUMORS USING L-3-[IODINE-123]IODO-ALPHA-METHYL-TYROSINE SPECT: COMPARISON WITH PROLIFERATIVE AND MITOTIC ACTIVITY, CELLULARITY AND VASCULARITY



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SUMMARY

The radiolabeled amino acid L-3-[^{123}I]-iodo-alpha-methyl-tyrosine (IMT) is a relatively new tumor tracer that accumulates in many tumors and is suitable for SPECT imaging. Using IMT SPECT, we studied 32 patients with a soft tissue tumor suspect for a soft-tissue sarcoma to determine whether (1) tumors can be visualized, (2) benign and malignant lesions can be distinguished and (3) IMT uptake is related to tumor grade and proliferation.

Imaging was performed 15 min after administration of 300 MBq IMT, biopsy or resection 1-2 weeks later. IMT uptake was quantified using a region-of-interest method resulting in tumor-to-background (T/B) ratios. These were compared with tumor grade, mitotic index, tumor cellularity, vascularity and the Ki-67 proliferation index.

Eleven patients had a benign tumor, 21 patients had a soft tissue sarcoma. Six benign tumors demonstrated minor IMT uptake, five lipomas had no uptake. All malignant tumors had high uptake and were clearly visualized. T/B ratios in malignant tumors (3.83±1.16) were higher ($p<0.001$) than in benign tumors (1.52±0.60). Small (<5 mm) metastases in two patients were not detected. Taking T/B ratio 2.0 as cut-off level, sensitivity for detection of malignancy was 100%, specificity 88%. IMT uptake correlated with histological grade ($r=0.82;p<0.001$), mitotic index ($r=0.75;p<0.001$), tumor cellularity ($r=0.73;p<0.01$) and with the Ki-67 proliferation index ($r=0.63;p<0.01$).

IMT SPECT visualized all soft-tissue sarcomas. Uptake in sarcomas was clearly higher than in benign lesions, yielding 100% sensitivity for the detection of malignancy at a specificity of 88%. Uptake increased with higher tumor grade and higher proliferation rate.

INTRODUCTION

Soft-tissue sarcomas are a heterogeneous group of malignant tumors that can arise from mesenchymal structures at any site of the body. These tumors constitute 1% of all cancers. A large proportion (60%) is located in an extremity. They often reach a large size before a diagnosis is established. They grow locally aggressive, frequently invade surrounding tissues and often disseminate to distant sites. Treatment planning depends on information regarding the presence or absence of metastases, the local situation and histological parameters. Chest CT is commonly used to screen for pulmonary metastases and MRI to assess the local situation and resectability. The most important prognostic factor is tumor malignancy grade, i.e. a higher tumor grade is associated with a worse prognosis [8,86,128,143].

Considerable interest exists in non-invasive determination of the malignancy grade of sarcomas [21,189-192]. For this purpose, various nuclear medicine techniques such as single photon emission computerized tomography (SPECT) and more recently positron emission tomography (PET) employing various tracers have been

applied. Radiolabeled amino acids are potentially suitable tracers: due to the increased protein metabolism of malignant tumors, uptake in malignancy is increased [193]. Furthermore tumor uptake is less disturbed by uptake in inflammatory tissue than the frequently used tracer ^{18}F-fluoro-2-deoxy-D-glucose (FDG) [174,194-197]. However, most carbon-11 labeled amino acids are difficult to synthesize and require the infrastructure of PET.

The radiolabeled amino acid L-3-[^{123}I]iodo-alpha-methyl-tyrosine (IMT) is avidly taken up in many tumors [198,199]. The agent has been introduced for imaging of brain tumors where tumor uptake was shown to represent amino acid transport activity, an important step in protein metabolism [200-203]. Since at present IMT is the only amino acid tracer suitable for SPECT, imaging with this tracer might combine the specificity of amino acids with the wide availability of SPECT.

The goals of this study were to determine (1) whether IMT SPECT can visualize soft-tissue sarcomas, (2) whether IMT uptake can discriminate between benign and malignant tumors and (3) whether uptake is related to tumor grade and the degree of proliferation, using the mitotic index and the Ki-67 proliferation index as markers.

PATIENTS AND METHODS

Patients

Patients referred to the University Hospital Groningen between October 1997 and February 1999 for management of a suspected soft-tissue sarcoma were asked to participate in this study. Suspicion for malignancy was based on rapid growth, size, consistency at palpation, location and radiographic appearance of the tumor. Selection criteria included age > 18 yr and planned histological confirmation within 2 weeks after the IMT study.

Thirty-two patients (16 male, 16 female; median age 45 yr (18-82) were included in the study. Patient and tumor data are presented in Table 1. All patients were studied 1-2 weeks prior to biopsy or definitive resection. All patients were clinically staged with a chest CT, MRI or CT of the soft tissue mass as well as ultrasound of the liver and bone scintigraphy. The study was approved by the Medical Ethics Committee of the University Hospital Groningen. Written informed consent was obtained from all patients.

Synthesis and Quality Control

Synthesis of IMT was carried out slightly modified from Krummeich et al. [204]. Briefly, Iodo-gen™ iodination with Na^{123}I (specific activity > 185 TBq/mmol (5000 Ci/mmol), Amersham Cygne, Eindhoven, the Netherlands) of the precursor L-alpha-methyl-tyrosine was performed in a borate buffer. IMT was purified by elution with saline containing 5% ethanol over a C-18 SepPak® cartridge (Waters, Milford, Mass. USA) preconditioned with 100% ethanol followed by saline containing 5% ethanol. After filtration through a sterile 0.22 μm Millex GV filter (Millipore®, S.A.,

Molsheim, France) a colorless ready-to-inject solution was obtained. Samples were demonstrated to be sterile and pyrogen-free. Quality control was performed by HPLC on a RP-18 column (Multosorb 100 4.6) using H_2O/ethanol/acetic-acid 87.5/10/2.5 v/v/v % as eluent. Radiochemical purity was over 99% in all cases. The overall synthesis time, including purification and quality control was less than 1 hr. Radiochemical yield was 50 - 65%.

Imaging

Patiens were injected intravenously with 200-300 MBq IMT after at least a 4 hr fast. They were given 10 drops of Lugol's solution orally 15 min prior to injection to prevent thyroid uptake of possibly formed free ^{123}I. A large-field-of-view double headed gamma camera (MULTISPECT 2, Siemens Inc, Hoffman Estates, Illinois, USA) was used with a medium energy all purpose collimator and a 15% window centered on the 159 keV photopeak of ^{123}Iodine. System resolution was 12 mm FWHM at 10 cm distance.

Fifteen minutes after injection SPECT of the tumor area was performed usually followed by a planar spot view. After imaging the tumor area, additional spot views were recorded to obtain whole body information in all cases. In 20 patients the thorax was imaged using SPECT.

All SPECT acquisitions included 64 views (2 x 32; 5.6 /step) of 30 seconds duration each in a 128 x 128 matrix format with a zoom factor of 1.23. This corresponds to a pixel dimension of 4.1 mm. Transaxial tomograms were reconstructed without prefiltering using filtered back-projection with a Butterworth filter of 6th order and a cutoff frequency of 0.275 Nyquist. Ten minutes spot views were recorded in a 128 x 128 matrix. Total imaging time was approximately 1 hr.

The reported radiation burden of IMT is 0.007 mSv/MBq, yielding an effective dose equivalent of 1.4 - 2.1 mSv [205].

Image analysis.

Without knowledge of the staging and histopathological data, two experienced readers analyzed all images visually for tumor uptake and abnormal extratumoral uptake. Regions of interest (ROIs) were manually placed over SPECT tumor slices with maximum visibility and on planar spot views. Reference ROIs were placed over representative background muscle tissue, usually contralaterally. ROIs were drawn at 80% of the maximal pixel value around the lesion under study, in some cases this procedure resulted in more than one ROI per tumor [206]. The T/B ratio was calculated by dividing tumor ROI intensity (cts/pixel) by background ROI intensity.

All IMT scintigraphic findings were compared with the results of conventional imaging. T/B ratios were compared with tumor grade, mitotic index, tumor cellularity and vascularity and the Ki-67 proliferation index.

Pathological examination

The histological diagnosis was made on haematoxilin-eosin (HE) stained paraffin sections with or without additional immunohistological stains. All tumors

were classified according to Enzinger and Weiss into 10 different histological types [8]. Soft-tissue sarcomas were graded according to the grading system of Coindre et al. in which points are assigned to differentiation level, mitotic index and necrosis [29].

In 27 of the 32 lesions determination of the mitotic index, Ki-67 proliferation index, cellularity and vascularity was performed. In five cases (3 benign, 2 malignant) there was not enough material left for these determinations. The number of mitotic figures per 2 mm^2 was counted in HE stained paraffin sections. Proliferating cells were detected using the monoclonal antibody MIB-1 (Immunotech S.A., Marseille, France), which recognizes an epitope of the Ki-67 antigen. This is a nuclear antigen present in all phases of the cell cycle except for the G0 phase, in which the cells are withdrawn from the cell cycle, and the early G1 phase, the phase before the start of DNA synthesis [170]. Immunohistochemistry was performed on paraffin sections according to a method modified from Shi et al. [177,178]. In short: after heating on a hot plate, the slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After heating twice in an autoclave for 10 minutes at 110 C in boiling solution pH=6.0, the slides were incubated with a 1:400 dilution of the antibody in bovine serum albumin pH=7.4. The primary antibody was detected with a biotinylated secondary antibody (multilink) followed by a streptavidine-alkaline phosphatase conjugate (Ready-to-Use Link and Label, Biogenex, San Ramon CA, USA). Final colour was developed using bromochloroindolyl-phosphate 4-nitroblue-tetrazolium-chloride (Boehringer, Mannheim, Germany).

To measure the Ki-67 proliferation index and tumor cellularity we used ocular micrometry on a Leica microscope (Rijswijk, the Netherlands) with an eyepiece grid at x400 magnification. Fifteen fields were randomly selected throughout histologically viable areas. Tumor cellularity (cellular density) was defined as the total number of cells excluding endothelial cells, inflammatory cells and necrosis and presented as number per 2 mm^2. MIB-1 positive nuclei were identified and the number of these nuclei was divided by the total number of nuclei in each of the 15 fields to calculate an index per field. The Ki-67 proliferation index (also named Ki-67 labelling index), representing proliferative activity, was defined as the mean of the indices of the 15 fields. Vascularity in each tumor was estimated by quantification of the total number of small blood vessels or parts of large vessels in HE stained paraffin sections. Ten fields were randomly selected and the total number of vessels in 10 fields at 400x magnification (corresponding to 2 mm^2) was counted.

Statistics

To determine the ability to distinguish malignant from benign tumors, a Student's t-test was performed on T/B ratios (normally distributed) in both groups. Spearman's correlation coefficient was used to correlate T/B ratios with Ki-67 proliferation index, mitotic index, tumor vascularity and cellularity. Kruskall Wallis non-parametric ANOVA with Dunnett's T3 post-hoc multiple comparisons test was performed to relate tumor grade with T/B ratios. Two-tailed p-values <0.05 were considered significant.

RESULTS

Eleven patients were found to have a benign lesion, the remaining 21 had a soft-tissue sarcoma. Three patients had previously been treated for a soft-tissue sarcoma (2,3 and 4 yrs earlier) and were now diagnosed to have a local recurrency. Eighteen tumors were localized in an extremity (56%), fourteen in the trunk (44%). Tumor size was between 2 and 23 cm, median 8.6 cm. There were 4 low grade (grade I), 5 intermediate grade (grade II) and 12 high grade sarcomas (grade III) (Table 1). Benign lesions were assigned grade 0.

All 21 soft-tissue sarcomas were visualized on the IMT SPECT images. Tumors in the extremities and in the chest were well visualized because of high tumor uptake and low background uptake, whereas tumors near excretory systems (kidneys, bladder, ureters) were more difficult to evaluate. IMT uptake in tumors was often inhomogeneous, in accordance with the typical histological appearance of sarcomas where areas of necrosis and hemorrhage are interspersed with areas containing vital tumor cells. A typical example is presented in Figure 1.

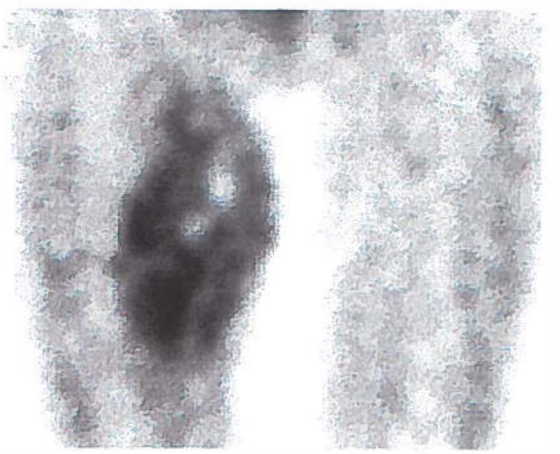


Figure 1.
Coronal SPECT slice through the upper legs of patient 13 with a high grade liposarcoma showing intense uptake of IMT. Heterogeneous uptake with a central defect is caused by necrosis.

In five of the eleven benign lesions no IMT uptake was found, in four faint uptake and in two avid uptake. No uptake was found in all five lipomas, faint uptake in two large intramuscular haemangiomas, an aspecific fibrotic lesion and in one aggressive fibromatosis lesion. Avid uptake was present in a growing neurofibroma and in another agressive fibromatosis lesions (these latter are considered 'borderline malignant').

Table 1. Patient characteristics and IMT uptake.

Pat	Age	Sex	Tumor histology	Location	size (cm)	grade	IMT T/B ratio
MALIGNANT LESIONS							
1	33	f	Liposarcoma (sclerosing)	upper leg	10	low	2.3
2	58	m	Liposarcoma	Retroperitoneal	23	low	2.6
3	32	f	Liposarcoma	upper leg	6	low	2.8
4	45	m	Liposarcoma (myxoid)	lower leg	13	low	2.9
5	47	f	Liposarcoma (myxoid)	knee	6	intermediate	3.8
6	36	m	Malignant fibrous histiocytoma	abdominal wall	8	intermediate	3.8
7	82	m	Malignant fibrous histiocytoma	lower leg	7	intermediate	5.4
8	43	m	Sarcoma not otherwise specified	upper leg	12	intermediate	4.0
9	35	f	Synovial sarcoma	upper leg	6	intermediate	3.2
10	18	m	Extra skeletal osteosarcoma	chest wall	5	high	2.7
11	29	m	Extraskeletal mesenchymal chondrosarcoma	elbow	2	high	4.1
12	58	m	Liposarcoma	chest wall	9	high	5.2
13	46	m	Liposarcoma (pleiomorphic)	upper leg	20	high	3.6
14	46	f	Malignant fibrous histiocytoma	upper leg	8	high	5.1
15	75	m	Malignant fibrous histiocytoma	upper leg	10	high	5.8
16	64	m	Malignant fibrous histiocytoma	chest wall	6	high	6.1
17	19	m	Malignant schwannoma	gluteal	7	high	3.2
18	18	f	Sarcoma not otherwise specified	gluteal	7	high	2.2
19	54	f	Sarcoma not otherwise specified	knee	10	high	3.5
20	66	m	Sarcoma not otherwise specified	knee	10	high	4.8
21	52	f	Synovial sarcoma	lower leg	10	high	3.4
BENIGN LESIONS							
22	24	f	Fibromatosis – locally invasive	neck	5		1.8
23	23	f	Fibromatosis – locally invasive	chest wall	6		2.5
24	41	m	Intramuscular haemangioma	back	2		1.5
25	42	f	Intramuscular haemangioma	lower leg	8		1.8
26	74	f	Lipoma	axilla	15		1.0
27	52	f	Lipoma	shoulder	6		1.0
28	56	m	Lipoma	upper leg	7		1.0
29	53	f	Lipoma	upper leg	8		1.0
30	42	f	Lipoma	back	5		1.0
31	28	m	Neurofibroma	groin	10		2.6
32	58	f	Non specific fibrosis	upper leg	10		1.5

The average T/B ratio in benign tumors was 1.52±0.60 whereas IMT uptake in the malignant tumors was much higher: 3.83±1.16. ($p<0.001$)(Figure 2). There was minimal overlap in individual values, due to avid uptake in the aggressive fibromatosis and in the growing neurofibroma (Figure 2). When a T/B ratio of 2.0 was chosen as cut-off point, sensitivity for the detection of malignancy in a soft-tissue mass was 100% with a specificity of 88%. Using 2.7 as cut-off point, sensitivity was 89% but specificity 100%.

T/B ratios correlated with histological grades ($r=0.82$, $p<0.001$): IMT uptake increased with higher tumor grade. Post hoc analysis revealed that the difference between benign tumors vs grade 1,2 and 3 tumors caused the significance. Grades 1 vs 2 and 2 vs 3 could not be distinguished from each other. However, low grade tumors could be discriminated from benign tumors in this group (Figure 3).

Interestingly, IMT uptake correlated with markers of cell proliferation: IMT T/B ratio vs the Ki-67 proliferation index gave $r=0.75$, $p<0.001$ (Figure 4A) and vs the mitotic index $r=0.63$, $p<0.01$ (Figure 4B). IMT uptake correlated also with tumor cellularity ($r=0.73$ $p<0.001$)(Figure 4C), while no relation with tumor vascularity could be established (Figure 4D).

Since whole body images were available in all patients, the ability of IMT scintigraphy to detect metastatic disease was also studied. But due to the low number of metastasized tumors no reliable assessment of staging could be obtained. Only 2 patients in this series were found to have metastatic disease: one had small pulmonary metastases (2 mm on chest CT) that were negative on the IMT chest SPECT study. The other patient had multiple small (<5 mm) intraperitoneal metastases, negative on all pre-operative investigations but detected during surgery.

DISCUSSION

This study demonstrates high uptake of IMT in soft-tissue sarcomas and low uptake in benign processes. All malignant tumors were visualized, leading to 100% sensitivity. Tumors in the direct vicinity of bladder or kidneys were somewhat more difficult to assess, due to renal excretion of IMT causing high background activity. IMT uptake could differentiate benign from malignant tumors with high accuracy. Uptake increased with higher tumor grade, higher Ki-67 proliferation index, mitotic activity and tumor cellularity while no correlation with tumor vascularity was found. Therefore, this relatively simple non-invasive technique provides information on parameters of tumor activity.

Only limited data were obtained to study whole body staging, since only two patients had metastases. In both patients these metastases were not detected. In general, lesion detectability depends on the resolution of the gammacamera and the amount of tracer uptake in a lesion: in this way a 1 mm lesion with very high tracer uptake can theoretically still be detected with a SPECT gammacamera with a resolution around 15 mm. In practice, however, most known SPECT tracers do not detect lesions smaller than 1.5 - 2 cm. Apparently this also holds true for the new

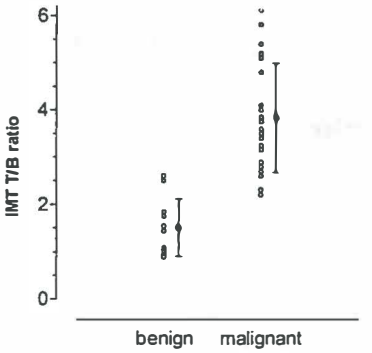


Figure 2.
IMT uptake (T/B ratios) in benign and malignant tumors (open symbols). Mean and SD (closed symbols). In malignant lesions uptake is higher than in benign lesions: 3.83±1.26 vs 1.52±0.60, $p<0.001$.

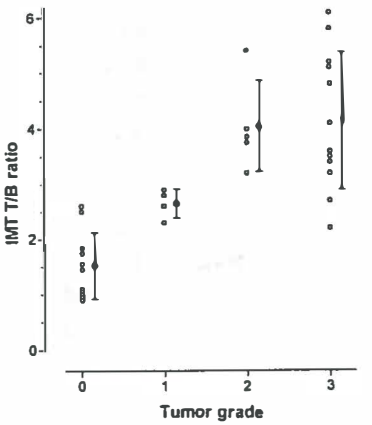
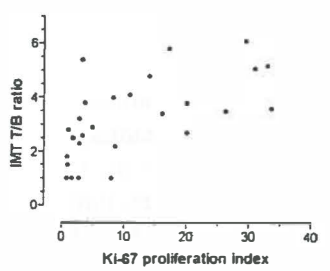
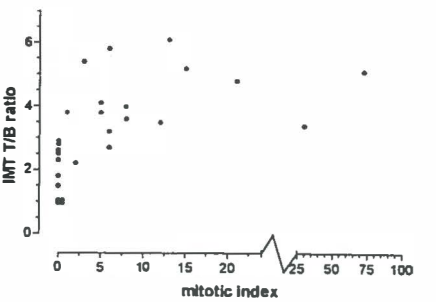
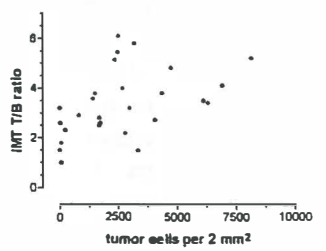


Figure 3.
IMT uptake (T/B ratio) versus tumor grade for all tumors (open symbols). Mean and SD (closed symbols). Benign tumors are assigned grade 0. Spearman correlation coefficient $r=0.82$, $p<0.001$.







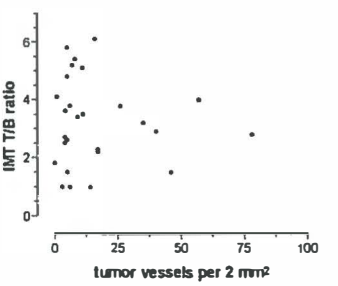


Figure 4
(A) Scatterplots showing IMT uptake (T/B ratio) in correlation with (A) the Ki-67 proliferation index: $r=0.63$, $p<0.01$ (B) mitotic index: $r=0.75$, $p<0.001$, (C) tumor cellularity: $r=0.73$, $p<0.001$, (4) tumor vascularity: no correlation.

tracer IMT: In our patient with lung metastases, lesions were probably too small and/or IMT uptake not high enough to enable detection. In the case of the patient with diffuse peritoneal metastases, both small size and interfering uptake in excretory organs (kidneys, ureters, intestine) probably prevented detection. This patient had a extra skeletal osteosarcoma of the chest wall. Also CT and MRI of the abdomen failed to detect the metastases. Although our experience in staging is very limited, it does not seems very likely that this method will contribute to improved staging due to above mentioned limitations.

To understand what processes are visualized by IMT uptake, it is important to understand the mechanism of uptake. Using competition experiments in vivo, Langen et al. demonstrated that IMT is a substrate for the amino acid transport systems in the blood-brain barrier [201]. This has not been demonstrated for tumors outside the brain yet. However, in vitro data suggest that also in non glioma tissue, uptake of IMT is almost entirely mediated by amino acid transport activity [207]. Since in the present study no correlation with the amount of tumor vessels was found, tumor perfusion is not the dominating factor that governs IMT uptake, as was suggested by Deehan in a rat model [208]. One could argue that uptake in the two haemangiomas must be related to blood flow and diffusion, but these tumors were also very cellular. The presumed mechanism of uptake of IMT is the accelerated protein metabolism and the resulting increased demand for amino acids. This is further supported by the relation with tumor proliferation factors, as found in this study.

To non-invasively determine the malignancy grade in soft-tissue tumors, magnetic resonance spectroscopy [192] and various nuclear medicine techniques and tracers have been used such as ^{201}Tl, ^{99m}Tc-MIBI, ^{99m}Tc-MDP, ^{67}Gallium [115,209] and more recently FDG and radiolabeled amino acids such as L-[1-^{11}C]-tyrosine [21,190,210,211]. Different biochemical processes in tumors are visualized by all these tracers: most likely ^{201}Tl uptake is related to cellular Na^+K^+-ATPase activity, ^{99m}Tc-MIBI to membrane functions and mitochondrial integrity, MDP to calcium/phosphate metabolism, ^{67}Ga to transferrine receptors in tumor cells or inflammatory cells. In all cases there is more or less effect of tumor vascularization. Sensitivity of tumor detection using IMT appears better than other single photon tracers in soft-tissue sarcomas, and the presumed uptake mechanism might more directly reflect tumor viability than the above mentioned mechanisms, but our results need to be confirmed by other studies.

Many studies have used PET to study sarcomas. FDG generally shows high uptake in tumors and has proven to be of value in the visualization and grading of soft-tissue sarcomas. In the study by Nieweg et al all 18 sarcomas were visualized using FDG PET and a correlation was found between the calculated glucose consumption and tumor grade [21]. Schulte et al found high sensitivity using FDG PET for the detection of malignancy in 102 patients, but lower specificity caused by high uptake in aggressive benign tumors and a patient with myositis ossificans [191]. Uptake of L-1-[^{11}C]-tyrosine also showed a correlation with tumor grade, mitotic rate and proliferation in soft-tissue sarcoma [190,211]. Because of lower uptake in inflammatory reactions caused by radiotherapy, systemic chemotherapy or regional cytostatic

perfusion, it is suggested that radiolabeled amino acids are better suited to monitor treatment effects than FDG [190,194,210]. The results of the present study using IMT SPECT are well in line with these PET studies, with somewhat higher correlation factors than reported for L-[1-^{11}C]-tyrosine [190,211].

Almost all studies on soft-tissue tumors have found some uptake in benign lesions, regardless of the used tracer [21,115,190,191,209]. Also using IMT we found minor uptake in haemangiomas and more avid uptake in a neurofibroma and in aggressive fibromatosis lesions. These latter are actually considered to be 'borderline malignant' and therefore presumably have increased amino acid metabolism leading to IMT uptake. Similar to our observations Kuwert et al found some IMT uptake in non-neoplastic brain lesions such as inflammatory lesions and infarctions) [212]. Recently very high uptake was described in a low-grade desmoplatic infantile ganglioglioma, another benign neoplasm [213]. Therefore, it seems most likely to conclude that IMT is an aspecific tumor tracer that targets amino acid metabolism in tumors. Difference in uptake between malignant and benign disease is based on the degree of accelerated protein metabolism.

In this study we found IMT uptake also to be related with the number of tumor cells. Whether this also holds true for other tracers is largely unknown. Since cellular density can be high in many tumors the mere fact of many cells per volume (in comparison with adjacent tissues) could already cause increased uptake . However, it seems unlikely that this effect only would results in T/B ratios between 3 and 6, as found in many high grade tumors. Moreover, others have found no relation between IMT uptake and cellular density in brain tumors [214].

Although our results appear comparable to those using FDG and L-[1-^{11}C]-tyrosine PET, it could be argued that the lower resolution and limited quantification possibilities of SPECT make the method inferior to PET. However, in these generally large tumors the lower resolution is presumably not a big problem, although partial volume effects may decrease tumor heterogeneity. Limited quantification possibilities are also controversial, since some authors successfully use T/B also in FDG PET studies [191], while others disagree as to what PET uptake parameters gives the best results in separation of benign from malignant disease [21,215-217]. Furthermore, absolute quantification of protein synthesis (as possible in PET) both in tumor tissue and in 'background' tissue may be disturbed by systemic influences and vary largely from day to day [210]. Using ratios might compensate for this effect.

The good correlation of IMT uptake with parameters of tumor activity may be of interest in evaluation of new treatment strategies such as angiogenesis inhibition, matrix metalloproteinase inhibition or antisense therapy . These new drugs exert their action through new mechanisms and may require new evaluation methods. In these methods assessment of metabolic tumor activity may complement the classic response parameter, the change in tumor size. In current routine clinical practice, however, non-invasive studies of tumor activity or its benign/malignant nature is of limited value. Patients and physicians rely on histological evidence, and only in exceptional cases these studies will influence diagnostic strategies.

In conclusion, IMT scintigraphy clearly visualized all soft-tissue sarcomas in this group. Uptake in malignant tumors was higher than in benign lesions, leading to a sensitivity for the detection of malignancy of 100% at a specificity of 88%. Uptake increased with higher tumor grade and higher proliferation rate. Therefore, IMT SPECT non-invasively provides information about tumor activity.

Acknowledgements
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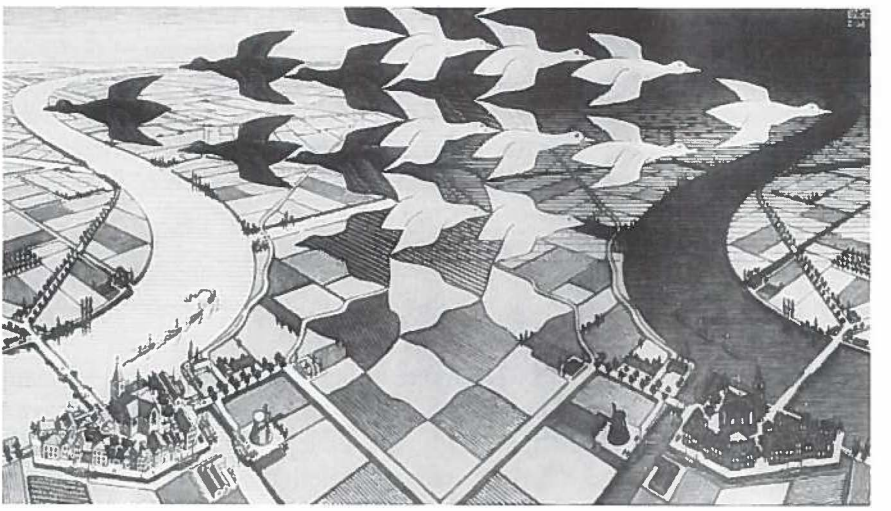
Chapter 5

HYPERTHERMIC ISOLATED LIMB PERFUSION WITH TNF-α AND MELPHALAN IN PATIENTS WITH LOCALLY ADVANCED SOFT TISSUE SARCOMAS: TREATMENT RESPONSE AND CLINICAL OUTCOME RELATED TO CHANGES IN PROLIFERATION AND APOPTOSIS



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SUMMARY

Hyperthermic isolated limb perfusion with tumor necrosis factor-α (TNF-α) and melphalan (HILP-TM) with or without interferon-γ (IFN-γ) is a promising local treatment in patients with locally advanced extremity soft tissue sarcomas (STS) with response rates of up to 84%. The mechanisms of the treatment response are poorly understood. This study determines the HILP-TM induced changes in mitotic activity, proliferation and apoptosis in 37 STS, the additional effect of IFN-γ and the relation with treatment response and clinical outcome.

On archival material, obtained before and 6-8 weeks after HILP-TM with TNF-α and melphalan with (n=15) or without (n=22) IFN-γ, the number of mitoses was counted and the proliferation fraction was determined by immunohistologic staining for the proliferation associated Ki-67 antigen (MIB1). Apoptosis was visualized by enzymatic detection of DNA fragmentation (TUNEL-method). . Clinical and histological response, follow-up status and survival were recorded.

The number of mitoses dropped 57% and proliferation rate decreased with 40% after HILP-TM, whereas the amount of apoptosis after HILP-TM more than doubled as before HILP-TM. The addition of IFN-γ to HILP-TM did not influence the changes in tumor parameters and did not affect treatment response. A better clinical response to HILP-TM was correlated with high mitotic activity and low amount of apoptosis in tumor samples before HILP-TM. Patients with highly proliferative STS before and after HILP-TM had a relatively poor prognosis. Furthermore, patients who developed distant metastases after HILP-TM had a relatively high number of dividing cells in the tumor remnants after treatment.

INTRODUCTION

Soft tissue sarcomas (STS) constitute a heterogeneous group of malignant tumors which arise from tissue of mesenchymal origin and account for approximately 1 % of all malignancies. STS predominantly affect the limbs and surgical resection in combination with high dose adjuvant external beam radiotherapy, after marginal resection, is the treatment of choice[8,218,219]. Because of the size and extent of the tumor or the proximity of vital structures, limb saving surgery is not always possible and mutilating surgical procedures or amputation are occasionally necessary[218,220-224]. One of the currently most promising limb saving procedures is the combination of hyperthermic isolated limb perfusion (HILP) with delayed surgical tumor excision[80,83]. The major advantages of HILP are that regional cytostatic concentrations can be 15 to 20 times as high as after systemic administration and that hyperthermia may enhance the cytotoxic effect[225,226]. In 1992 Lienard et al. used HILP to administer high dose recombinant tumor necrosis factor alfa (TNF-α) in combination with melphalan, an alkylating chemotherapeutic agent, resulting in encouraging outcomes [81]. In HILP with TNF-α and melphalan (HILP-TM) with or without interferon-gamma (IFN-γ) response rates of up to 84% have been reported in

the treatment of STS [79-83]. Since the mechanism of the antitumor effects of HILP-TM is still poorly understood and studies suggest that chemotherapeutic drugs in general inhibit proliferation by generating cell cycle arrest and subsequent apoptosis, it was decided to investigate these tumor parameters in HILP-TM [227-234].

The objective of this study was to investigate whether the treatment response and clinical outcome of patients treated with HILP-TM is associated with tumor grade, mitotic activity, proliferation and apoptosis in the tumor specimens of STS obtained before and after HILP-TM. Furthermore, the influence of HILP-TM on these tumor markers and additional effect of IFN-γ was evaluated.

PATIENTS AND METHODS

Patients

The inclusion criteria for the present study were: (1) histological diagnosis of a malignant mesenchymal tumor, located in the soft tissues; (2) primary irresectable tumor of lower or upper limb which was treated with HILP-TM with or without IFN-γ and (3) the availability of paraffin embedded tumor material obtained within 3 months before HILP-TM with a maximum period between pretreatment and posttreatment tumor samples of 5 months.

Between 1991 and 1997, 37 patients with primary irresectable STS of lower or upper limb underwent HILP-TM with TNF-α and melphalan. HILP-TM with TNF-α and melphalan was combined with IFN-γ in 15 of the 37 analyzed patients. Mean age of the 18 men and 19 women, at the time of diagnosis, was 46 (range: 18 - 18; median: 48; SD: 16.7) years. Four tumors were located in the upper limb (11%) and 33 in the lower limb (89%). At the time of HILP-TM, 6 patients (16%) had distant metastases and 3 patients (8%) had regional lymph node metastases. The mean time between incisional biopsy and perfusion was 22 (range: 0 - 83; median: 17; SD: 17) days. In one patient an incisional biopsy of multiple superficial sarcomas was performed at the day of HILP-TM to obtain fresh tumor material. In 35 of the 37 patients HILP-TM was followed by surgical resection 6-8 weeks later, according to the study protocol. In two patients (5%) histological examination after HILP-TM was not possible: one patient had progressive lung metastases requiring chemotherapy and in the other patient the limb had to be amputated because of vascular occlusion 2 days after HILP-TM. Local progressive disease or treatment related morbidity were reasons to perform the delayed local surgical resection earlier or later than planned in the protocol. Specimens to assess histological reaction were obtained after a mean period of 61 days following HILP-TM (range: 12 - 103; median: 60; SD:16). In three patients, in which HILP-TM was performed twice, histological tumor reaction was assessed after the first HILP-TM in two patients and after the second HILP-TM in one patient, of whom no tumor material was available after the first HILP-TM. The mean time between the pre-treatment sample and the post-treatment sample was 83 days (range: 33 - 138; median: 80; SD: 22). No additional treatment was given

between HILP-TM and the delayed resection. Patient characteristics, histology and clinical data are presented in Table 1.

Histology

In all cases the histological diagnosis was made on H&E stained paraffin sections of incisional biopsies with or without additional immunohistological stains. All cases were classified according to Enzinger and Weiss [8] which revealed 16 different histological types (Table 1). The STS were graded according to the grading system of Coindre et al., in which points are assigned to differentiation level, mitotic index and necrosis [29]. This resulted in 7 grade I (19%), 19 grade II (51%) and 11 grade III (30%) STS (Table 1). In the resection specimens the amount of necrosis was estimated on gross examination. For histology at least one section per centimeter of the largest tumor diameter was taken. Care was taken to histologically document the presence of necrosis, viable tumor or fibrosis.

Tumor parameters

The following tumor parameters were studied on adjacent slides of the macroscopically and microscopically most viable parts of the tumor before and after HILP-TM: mitotic index, proliferation and apoptosis.

The number of *mitoses* per 2 mm^2 (mitotic index) in adjacent fields was counted on H&E stained paraffin sections.

For *proliferation*, the monoclonal antibody MIB 1 (Immunotech S.A., Marseille, France) was used. This antibody recognizes an epitope of the Ki-67 antigen, which is present in the nucleus of cells in all phases of the cell cycle except the G0 and the early G1 phase [170]. Immunohistochemistry was performed on paraffin sections (4 µm) according to a method modified from Shi et al.[177,178]. Briefly, after heating on a hotplate slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After two times heating in an autoclave for 10 minutes at 110° C in a 20 mM blocking reagens (Boehringer Mannheim, Mannheim, Germany) with pH=6.0, slides were incubated with a 1:400 dilution of the antibody in bovine serum albumin buffer pH=7.4. The primary antibody was detected with a biotinylated secondary antibody (multilink) followed by a streptavidine-alkaline phosphatase conjugate (Ready-to-Use Link and Label, Biogenex). Final color was developed by the BCIP-NBT method (bromochloroindolyl-phosphate 4-nitroblue-tetrazoliumchloride; Boehringer Mannheim, Mannheim, Germany).

Apoptosis was studied in 4 µm sections of formaldehyde fixed and paraffin embedded tissue using the TUNEL (Terminal deoxynucleotidyl Transferase (TdT) mediated dUTP Nick End Labeling)-method. After deparaffination, sections were subjected to RNA-se (1 mg/ml in 50 mM Tris-HCl and 150 mM NaCl pH 7.5; Aldrioch , USA) treatment for 30 minutes at room temperature to prevent non-specific binding digoxygenin labeled deoxy UTP (DIG-11-dUTP). Slides were subsequently incubated with 15 µl of a 100 µl solution containing Terminal deoxynucleotidyl transferase (TdT) buffer (Pharmacia, Uppsala, Sweden) with 5 mM cobalt chloride and 1% bovine serum albumin (Aldrich, USA) together with 5 nM

DIG-11-dUTP (Boehringer Mannheim, Mannheim, Germany) for 60 minutes at 37 ° C. After this reaction, slides were rinsed twice in a 0.1x standard sodium citrate solution for 15 minutes at room temperature. Non-specific background staining was prevented by preincubating slides with 5% blocking reagens (Boehringer). Sections were incubated overnight at 6 ° C with alkaline-phosphatase labeled sheep anti-DIG FAB fragments diluted 1:300 in 2% blocking reagens (Boehringer). After extensive washing, alkaline-phophatase was visualized with nitroblue tetrazolium 5-brome-4-chromo-3-indolyl-phosphatase (Boehringer) for 50 minutes at room temperature. Sections were counterstained with haematoxylin and coverslipped with a mounting medium soluble in xylene.

Quantification of Ki-67 and apoptosis

All sections stained for proliferation and apoptosis were quantified without knowing whether this section was before or after HILP-TM. For measuring the Ki-67 labeling index (LI) and the apoptosis LI we used ocular micrometry on a Leitz microscope by using an eyepiece grid at x400 magnification. Fifteen adjacent fields in histologically viable areas were quantified. The positive and negative nuclei were counted. Endothelial cells, inflammatory cells and necrosis were excluded. The number of positive nuclei was then divided by the total number of nuclei in each of the fifteen randomly selected fields to calculate an index per field. The Ki-67 LI (representing proliferative activity) and the apoptosis LI (representing apoptotic activity) were defined as the mean of the indices of the fifteen randomly selected fields.

Drugs and Treatment Schedule

The technique of HILP with TNF-α and melphalan with or without IFN-γ for upper and lower limb was recently extensively described[80,83]. Briefly, recombinant human TNF-α (0.2 mg per ampule, 4.9 to 5.8 x 10^7 U/mg per ampule, Boehringer Ingelheim, GmbH, Ingelheim/Rhein, Germany) and, in 16 patients, recombinant human IFN-γ (0.2 mg or 1.5 x 10^6 U per ampule, Boehringer Ingelheim, GmbH, Ingelheim/Rhein, Germany) was used. The cytostatic drug melphalan (Alkeran®) was obtained as a sterile powder (100 mg) that was dissolved aseptically using solvent and diluent as provided by Glaxo Burroughs Wellcome (London, United Kingdom). After isolation of the systemic blood circulation under general anaesthesia and with heparinization (3 mg/kg), the limb was perfused with 3 mg (upper limb) TNF-α to 4 mg TNF-α (lower limb) administered as a bolus and, after 30 minutes, with 13-mg/L (upper limb) or 10-mg/L (lower limb) volume of melphalan at 39 to 40° C with or without addition of 0.2 mg IFN-γ. The HILP treatment with TNF-α and melphalan with or without IFN-γ was approved by the local medical ethical committee of the Groningen University Hospital.

Assessment of Tumor Response

Tumor response was assessed as previously described by the European TNF perfusion group[80,83]. To estimate the tumor response, both clinical and histological reaction on HILP-TM were validated. Briefly, the clinical response was regularly determined by standard physical and radiodiagnostic examination i.e. magnetic resonance imaging or computed tomography during the 6-8 weeks period between HILP-TM and protocol planned delayed resection. The largest diameter of the tumor in centimeters before and after HILP-TM was used as the clinical parameter. Clinical complete response (CR) was defined as the disappearance of all measurable disease in the limb for longer than 4 weeks; clinical partial response (PR) was defined as a regression of the tumor size >50% for longer than 4 weeks. A regression of the tumor size <50% or a progression of the tumor size <25% for longer than 4 weeks was interpreted as clinical no change (NC), whereas clinical progressive disease (PD) was defined as a progression of the tumor size >25% for longer than 4 weeks. Histological CR was defined as 100% necrosis after HILP-TM, histological PR as ≥50% and <100% necrosis and histological NC as ≥0% and <50% necrosis. Overall response was determined as the combination of both clinical and histological tumor response. A clinical CR was downgraded to overall PR when there was a histological PR or NC. Otherwise, a clinical PR could be upgraded to overall CR when a histological CR was observed. In addition, a clinical tumor regression of <50 % was upgraded to overall PR when a histological PR or CR was present. When a clinical PR was observed but the histological examination revealed <50 % necrosis (NC), the overall response was considered as a PR.

Statistics

A Mann Whitney Test was carried out to analyze the differences in tumor parameters before and after HILP-TM of all 37 studied patients. A Wilcoxon matched pairs signed rank sum test was used to analyze the differences in tumor parameters before and after HILP-TM of the 29 patients with viable tumor both before and after HILP-TM. To quantitate the degree of correlation between parameters, the Spearman's rank test was used. A two-tailed p-value of < 0.05 was considered to be significant. Statistical analysis was performed using Graphpad Prism 2.0 (Graphpad Software Inc., San Diego, California, USA). Actuarial survival curves were constructed by the Kaplan-Meier method in order to compare OS and DFS for the different patient groups in relation to HILP-TM modality as well as high (i.e. above the median value) or low (i.e. smaller or equal to the median value) tumor parameters. Survival curves in the different groups were compared by the log-rank test using SPSS 8.0 for Windows.

Table 1. Patient characteristics, histology, HILP-TM-modality, (clinical, histological and overall) treatment response and clinical outcome.

Pt	age	sex	limb	Diagnosis	grade	Stage	+/- IFN	Clin. Resp.	Hist. Resp.	Overal l Resp.	LR (mth)	Meta mth)	FU (mth)	
1	47	F	lower	MFH	I	**	+	PR	CR	CR	-	-	NED	46
2	37	M	lower	Myxoid LPS	I	IB	-	PR	PR	PR	-	-	NED	11
3	44	M	lower	Myxoid LPS	I	IB	-	PR	PR	PR	-	-	NED	31
4	44	F	lower	Myxoid LPS	I	IB	+	PR	PR	PR	-	-	NED	55
5	48	F	lower	Well diff. LPS	I	IB	+	NC	NC	NC	-	-	NED	48
6	49	M	lower	Mal.Hemang.	I	IB	-	PR	PR	PR	-	-	NED	17
7	42	M	lower	Clear cell sarc.	I	IVA	-	PR	PR	PR	-	10	DOD	23
8	18	M	lower	Extrask myxoid chondrosarcoma	II	IIB	+	PR	PR	PR	-	-	NED	55
9	18	F	lower	RMS	II	IIB	+	PR	CR	CR	-	-	NED	59
10	24	M	lower	Synovial sarc.	II	IIB	-	CR	PR	PR	-	-	NED	32
11	37	F	upper	MPNST	II	IIB	-	PR	PR	PR	-	-	NED	26
12	43	M	lower	Synovial sarc.	II	IIB	+	NC	NC	NC	-	35	NED	55
13	48	M	lower	Myxoid LPS	II	IIB	-	NC	PR	PR	-	-	NED	17
14	50	F	lower	Sarcoma NOS	II	IIB	-	CR	CR	CR	-	-	NED	36
15	53	M	lower	MFH	II	IIB	+	PR	PR	PR	-	7	DOD	17
16	53	F	lower	Sarcoma NOS	II	IIB	-	PR	CR	CR	-	-	NED	27
17	56	F	lower	PPNET	II	IIB	-	PR	CR	CR	9	-	NED	43
18	61	F	upper	MFH	II	IIB	+	PR	PR	PR	-	-	NED	64
19	62	M	lower	MPNST	II	IIB	+	NC	NC	NC	-	5	DOD	16
20	64	F	lower	MPNST	II	IIB	-	PR	PR	PR	4	-	NED	43
21	66	M	lower	MFH	II	IIB	-	PR	PR	PR	-	6	DOD	28
22	80	M	lower	MFH	II	IIB	-	PR	NC	PR	-	8	DOD	10
23	71	M	lower	LPS	II	IIB	+	NC	PR	PR	-	13	AWD	59
24	21	M	upper	Epith. Sarcoma	II	IVA	-	PR	PR	PR	1	9	AWD	14
25	22	M	lower	Epith. Sarcoma	II	IVA	+	PR	NC	NC	6	22	DOD	40
26	33	F	lower	LMS	II	IVB	-	PR	CR	CR	-	Bef	DOD	8
27	25	F	lower	Synovial sarc.	III	IIIB	-	PR	PR	PR	-	-	NED	38
28	28	M	lower	Sarcoma NOS	III	IIIB	+	CR	NC	PR	-	Unc	DOD	18
29	39	F	upper	Synovial sarc.	III	IIIB	-	CR	PR	PR	-	23	AWD	23
30	60	F	lower	LMS	III	IIIB	+	PR	PR	PR	-	15	DOD	38
31	67	F	lower	MFH	III	IIIB	-	PR	NC	PR	3	-	DOD	9
32	69	F	lower	Fibrosarcoma	III	IIIB	-	PR	PR	PR	-	-	NED	28
33	74	M	lower	Sarcoma NOS	III	IIIB	-	PR	PR	PR	3	11	DOD	11
34	50	F	lower	Dediff. LPS	III	IVB	+	PR	PR	PR	-	Bef	DOD	9
35	26	F	lower	Sarcoma NOS	III	IVB	-	PR	UNK	UNK	-	Bef	DOD	7
36	40	F	lower	LMS	III	IVB	+	PR	PR	PR	-	Bef	DOD	9
37	54	M	lower	MFH	III	IVB	-	CR	UNK	UNK	-	Bef	DOD	3

HILP-TM: hyperthermic isolated limb perfusion with TNF-α and melphalan, +/- IFN: with or without IFN-γ, LR: local recurrence, meta: distant metastases, mth: months, FU: follow-up, sarc.: sarcoma, mal.: malignant, Hemang.: hemangiopericytoma, LMS: leiomyosarcoma, RMS: rhabdomyosarcoma, MPNST: malignant peripheral nerve sheath tumor, MFH: malignant fibrous histiocytoma, pPNET: peripheral primitive neuroectodermal tumor, NOS: not otherwise specified, IFN: Interferon gamma, CR: complete response, PR: partial response, NC: no change, PD: progressive disease, NED: no evidence of disease, AWD: alive with disease, DOD: dead of disease, UNK: unknown, Bef: before, Unc: uncertain, patient developed a 2nd malignant tumor.

RESULTS

HILP-TM with or without IFN-γ

As shown in Table 2, tumor grade, mitotic activity, amount of proliferation and apoptosis before and after HILP-TM of the 15 STS treated with HILP-TM in combination with IFN-γ did not differ from the 22 STS treated with HILP-TM alone. Furthermore, the changes in these tumor parameters after HILP-TM as compared to pre-treatment values did not differ significantly between both groups. Treatment responses, clinical outcome and survival did not differ between the IFN-γ treated patient group and the patients who received HILP-TM without IFN-γ. Therefore it was decided to perform the statistical analyses on the whole group.

Table 2. Tumor parameters of tumors treated with HILP-TM with or without IFN-γ

		HILP-TM with IFN-γ (mean ± SD) n = 15	HILP-TM without IFN-γ (mean ± SD) n = 22	p-value
Before HILP-TM:	viable tumor (%)	85 (± 23)	86 (± 22)	>0.05
	mitotic count (/2 mm^2)	13 (± 17)	12 (± 13)	>0.05
	proliferation (%)	16.6 (± 14.5)	18.4 (± 11.2)	>0.05
	apoptosis (%)	1.06 (± 1.02)	0.80 (± 0.68)	>0.05
After HILP-TM:	viable tumor (%)	45 (± 34)	27 (± 21)	>0.05
	mitotic count (/2 mm^2)	4 (± 5)	8 (± 16)	>0.05
	proliferation (%)	9.8 (± 9.2)	12.7 (± 12.2)	>0.05
	apoptosis (%)	2.58 (± 2.40)	1.68 (± 2.56)	>0.05
Δ viable tumor (%)		- 42 (± 35)	- 61 (± 28)	> 0.05
Δ mitotic count (/2 mm^2)		- 8 (± 18)	- 6 (± 17)	> 0.05
Δ proliferation (%)		- 7.6 (± 8.1)	- 6.1 (± 10.0)	> 0.05
Δ apoptosis (%)		+ 1.57 (± 2.51)	+ 0.91 (± 2.67)	> 0.05
overall response	complete response	2 (13 %)	4 (20 %)	
	partial response	9 (60 %)	16 (80 %)	
	no change	4 (27 %)	0	
mean OS (months)		44	31	0.83

Δ: absolute change, HILP-TM: hyperthermic isolated limb perfusion, IFN-γ: Interferon gamma, OS: overall survival

Treatment response related to tumor parameters before HILP-TM

A clinically assessed complete response was observed in 5 patients (14%), a partial response was noticed in 27 patients (73%) and in 5 patients (14 %) no response could be detected clinically (Table 1). After histological evaluation, the overall tumor response after HILP-TM was considered to be complete in 6 patients

(16%) and partial in 23 patients (62%). In six patients (16%) the tumor did not respond and in two patients the histological response could not be evaluated. Both mitotic rate and the amount of apoptosis before HILP-TM did correlate ($p<0.05$) with the clinical response (r: 0.41 and r: -0.33, respectively), i.e. high mitotic index and low amount of apoptosis before HILP-TM was associated with a relatively good clinical response. However, these parameters did not correlate with the histological or overall response. Tumor grade and amount of proliferation before HILP-TM did not significantly correlate with clinical, histological or overall response, although a nearly significant correlation (r: 0.31; $p=0.06$) between tumor grade and clinical response was found i.e. clinical response was relatively good in high grade tumors. The clinical, histological or overall response were not associated with the period between HILP-TM and the delayed local excision.

HILP-TM induced changes in tumor parameters

The changes in tumor parameters in tumors of individual patients could be assessed before and after HILP-TM in the 29 patients with viable tumor both before and after HILP-TM (Figures 1a-c). Changes in tumor markers or post-treatment values did not correlate with the period between HILP-TM and the collection of the post-treatment samples nor between the time between both samples. The mean number of *mitoses* in these patients declined from 14 before HILP-TM to 6 after HILP-TM ($p<0.01$) (Table 3). The changes in mitotic index were correlated (r: 0.47; $p<0.05$) with the clinical response, but not with the histological or overall response. Furthermore, no correlation with other clinical or tumor markers was found. In 4 out of 29 patients the number of mitoses after HILP-TM was elevated as compared to before HILP-TM. The two patients with the highest increase developed distant metastases after HILP-TM.

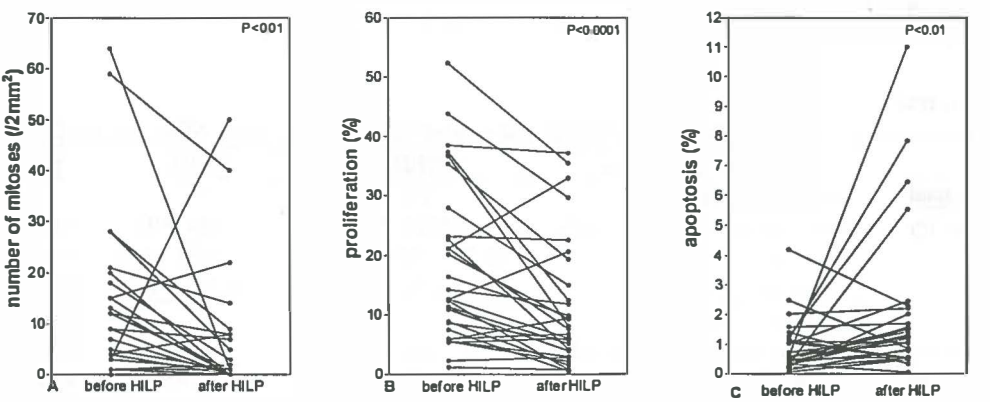


Figure 1
Paired analysis of the studied tumor parameters (**A**: mitotic count, **B**: proliferation, **C**: apoptosis) of the 29 patients with viable tumor both before and after HILP-TM.

Table 3: Tumor parameters *before* and *after* HILP-TM

Tumor parameter	n	Before HILP-TM (mean±SD)	after HILP-TM (mean±SD)	p
Mitotic count (/2mm²)	29	14 (± 16)	6 (± SD: 12)	< 0.01
Proliferation	29	18.2 % (± 13.6 %)	11 % (± SD: 10.8 %)	<0.0001
Apoptosis	29	0.87 % (± 0.86 %)	2.09 % (± SD: 2.49 %)	<0.01

Mean *proliferative activity* dropped significantly ($p<0.001$) from 18.2 % before HILP-TM to 11.0 % after HILP-TM. As shown in Figure 1b, 23 patients had a decrease in proliferation, whereas 6 patients had an increase. The two patients with the largest increase in proliferation developed distant metastases and died of disease. However, there was no significant correlation between the change in proliferation and other tumor or clinical parameters. The mean percentage of *apoptotic cells* increased from 0.87 % before HILP-TM to 2.09 % after HILP-TM ($p<0.01$). In seven patients (Figure 1c), a decrease in apoptosis was observed, including two patients who had an increase in proliferation after HILP-TM. The increase in apoptosis was not correlated with other tumor or clinical parameters.

Follow-up, metastasis and survival in relation to the studied tumor parameters

After a mean follow-up period of 29 months (range: 3 - 64; median: 27; SD: 17.7) months after HILP-TM, 19 patients were alive without evidence of disease (NED), 3 patients were alive with disease (AWD) and 15 patients died of disease (DOD) (Table 1). In the tumors of the patients who died of disease, a higher amount of mitoses and proliferation was observed ($p<0.05$) both *before and after* HILP-TM as compared to the patients who were alive (Table 4) . Amount of apoptosis (before or after) did not differ between those groups. The change in mitotic, proliferative and apoptotic activity after HILP-TM was not related to follow-up status.

Table 4.
Tumor parameters *before* and *after* HILP-TM related to follow-up status NED and DOD

	Tumor parameter	NED	DOD	p
Before	number of patients	19	15	
HILP:	mean mitotic count (/2mm²)	7 (SD: 7)	20 (SD: 19)	0.01
	mean proliferation	14.7 % (SD: 13.1)	22.3 % (SD: 11.8)	0.04
	mean apoptosis	0.92 % (SD: 0.65)	0.74 % (SD: 0.62)	0.41
After	number of patients	14	12	
HILP:	mean mitotic count (/2mm²)	2 (SD: 3)	13 (SD: 17)	0.02
	mean proliferation	8.0 % (SD: 9.6)	16.0 % (SD: 11.5)	0.02
	mean apoptosis	2.72 % (SD: 3.27)	1.56 % (SD: 1.38)	0.70
change:	mean Δ mitotic count (/2mm²)	-5 (SD: 8)	-7 (SD: 25)	0.55
	mean Δ proliferation	-7.2 % (SD: 8.8)	-7.5 % (SD: 9.7)	0.69
	mean Δ apoptosis	1.85 % (SD: 3.29)	0.89 % (SD: 1.53)	0.94

Table 5. Tumor parameters *before* and *after* HILP-TM related to newly developed distant metastases after HILP-TM

	Tumor parameter	distant metastases	no distant metastases	p
Before	number of patients	12	24	
HILP:	mean mitotic count (/2mm²)	15 (SD: 16)	10 (SD: 9)	0.32
	mean proliferation	20.8 % (SD: 12.5)	16.2 % (SD: 12.7 %)	0.22
	mean apoptosis	0.92 % (SD: 1.12)	0.93 % (SD: 0.68%)	0.56
After	number of patients	12	16	
HILP:	mean mitotic count (/2mm²)	12 (SD: 17)	2 (SD: 4)	0.05
	mean proliferation	13.6 % (SD: 11.3)	10.1 % (SD: 10.9 %)	0.34
	mean apoptosis	1.26 % (SD: 0.71)	2.79 % (SD: 3.17%)	0.53
change:	mean Δ mitotic count (/2mm²)	-1 (SD: 17)	-6 (SD: 8)	0.98
	mean Δ proliferation	-7.2 % (SD: 6.9)	-6.5 % (SD: 10.9 %)	0.50
	mean Δ apoptosis	0.32 % (SD: 0.91)	1.91 % (SD: 3.26 %)	0.44

Six patients (16 %) developed a local recurrence or regional lymph node metastasis after a mean disease free survival (DFS) of 4.3 (range: 1 - 9; median: 3.5; SD: 2.8) months. Twelve of the 31 patients (39 %) without distant metastases at the time of HILP-TM developed distant metastases after a mean DFS of 13.7 (range: 5 - 35 ; median: 11; SD: 8.9) months. In one patient with a sarcoma not otherwise specified, who developed a malignant tumor 11 months after HILP, it was uncertain whether this was a metastatic lesion or a second malignant primary tumor. Tumor grade, mitotic activity, proliferation or apoptosis before HILP-TM were not related to the development of metastases. However, in the patients who developed new distant metastases after HILP-TM the number of mitoses in the post HILP-TM specimens was higher ($p<0.05$) as compared to that in the patients who did not (Table 5). In contrast, proliferation and apoptosis after HILP-TM were not significantly associated with development of distant metastases. Development of metastatic disease or clinical outcome were neither associated with the time between HILP-TM and the delayed local resection nor with the period between the pre-treatment an posttreatment samples.

The 2 years OS for the 37 HILP-TM treated patients was 62 % and 5 years OS was 51%. Mean OS of the patients with a clinical CR was 51 months (PR: 42 months, NC: 42 months). Patients with a complete overall response had a mean OS of 51 months, which differed ($p=0.12$) from patients who had no response (mean OS: 29 months). Mean OS of patients with an overall PR was 47 months. As shown in Table 6, large (i.e. above the median) or small (i.e. below or equal to the median) changes in mitotic rate, proliferation or apoptosis after HILP-TM did not influence OS. However, the patients with a high amount of proliferation after HILP-TM had a shorter ($p<0.05$) OS than the patients with a low amount of proliferation after HILP-TM. The amount of mitotic rate or amount of apoptosis did not influence OS.

Table 6. Mean overall survival in relation to tumor parameters *before* and *after* HILP-TM

	Tumor parameter	mean OS of patients with low values (< or = the median)	mean OS of patients with high values (> the median)	p
Before HILP:	mean mitotic count (/2mm²)	50 months	32 months	0.07
	mean proliferation	48 months	34 months	0.04
	mean apoptosis	34 months	48 months	0.31
After HILP:	mean mitotic count (/2mm²)	45 months	37 months	0.23
	mean proliferation	49 months	33 months	0.02
	mean apoptosis	37 months	43 months	0.94
change:	mean Δ mitotic count (/2mm²)	38 months	38 months	0.91
	mean Δ proliferation	37 months	46 months	0.24
	mean Δ apoptosis	46 months	35 months	0.40

DISCUSSION

Since it was first described by Creech *et al.*in 1958, HILP is predominantly used in the treatment of melanomas and sarcomas[81,82,235-242]. Traditional cytostatic agents like melphalan and doxorubicin yielded unsatisfactory response rates after HILP for STS[243-247]. HILP with TNF-α and melphalan with or without interferon-gamma (IFN-γ) has dramatically improved the local tumor control of primary irresectable STS and response rates of up to 84%, resulting in preservation of the perfused limb without major dysfunction in over 80 % of the patients, have been reported [79-83]. This study did correlate tumor markers with treatment response and clinical outcome. It was found that a good clinical response was associated with high mitotic index, high tumor grade and low amount of apoptotic cells before treatment. A relation between tumor grade and treatment response was also suggested by Eggermont *et al.*who noted that high grade tumors grade tend to respond better to HILP-TM[83]. Tumor grade is determined by differentiation level, necrosis and number of mitoses. The latter seems to be more associated with clinical response than amount of necrosis or degree of differentiation, since also the proportion of the shift in mitotic index was correlated with clinical outcome. The pretreatment apoptosis values were positively correlated with clinical treatment response and negatively correlated with pretreatment mitotic rates. This might indicate that the response to HILP-TM is enhanced in STS with an apparently disturbed balance between cell growth and programmed cell death. The absence of a correlation between the studied tumor markers before HILP-TM and the histological or overall tumor response can be explained by the differences between the clinical and histological assessments. The clinical evaluation of tumor regression depends on the estimated size of the perfused tumor which is measured several times during the period between HILP-TM and the delayed resection by both radiodiagnostic and physical examination. The relatively late definitive histological examination 6-8 weeks after HILP-TM is carried out on the samples after HILP-TM representing the nonresponding, viable parts of

the tumor. Furthermore, the way of assessing histological treatment response could also explain the lack of correlation since it is based on the macroscopically and microscopically estimated percentage of necrosis after HILP-TM without taking into account the amount of necrosis before HILP-TM. Moreover, tumor parameters could not be studied in the post HILP-TM samples of 8 patients.

The additive effect of IFN-γ on HILP-TM is subject of debate [83]. In this *in vivo* study the changes in proliferation or cell death after HILP-TM did not differ between the patients treated with or without IFN-γ. Furthermore IFN-γ did not influence the changes in tumor parameters, nor treatment response or clinical outcome. It can be concluded from this study that the addition of IFN-γ in HILP-TM for STS does not substantially affect treatment response or clinical outcome.

The biological mechanisms which induce changes in tumor parameters during the 6-8 weeks between HILP-TM and resection remain unclear. Several authors postulated vascular occlusion as a result of intravascular coagulation after endothelial damage by TNF-α followed by hemorrhagic necrosis [248-251]. Since animal models and in vitro studies have reported the synergistic effect of TNF-α and melphalan resulting in cell death and tumor regression, some authors have postulated a more complex process in which vascular endothelium of the tumor, adhesion molecules and direct cytotoxic effects of melphalan and/or TNF-α may play a role [252-258]. This clinical study shows a significant decrease in number of both dividing and proliferating cells as well as an increase in apoptosis after HILP-TM. It remains unclear, however, whether the observed changes in proliferation and apoptosis are a secondary phenomenon occurring 6-8 weeks after HILP-TM induced necrosis, or the remnants of a massive chemotherapeutically generated programmed cell death induced by cell cycle blockage with or without DNA damage [257]. However, the significant increase in number of apoptotic cells *after* HILP-TM indicates a more subtle process of cell death induced by TNF-α and/or melphalan, than hemorrhagic necrosis after vascular occlusion alone.

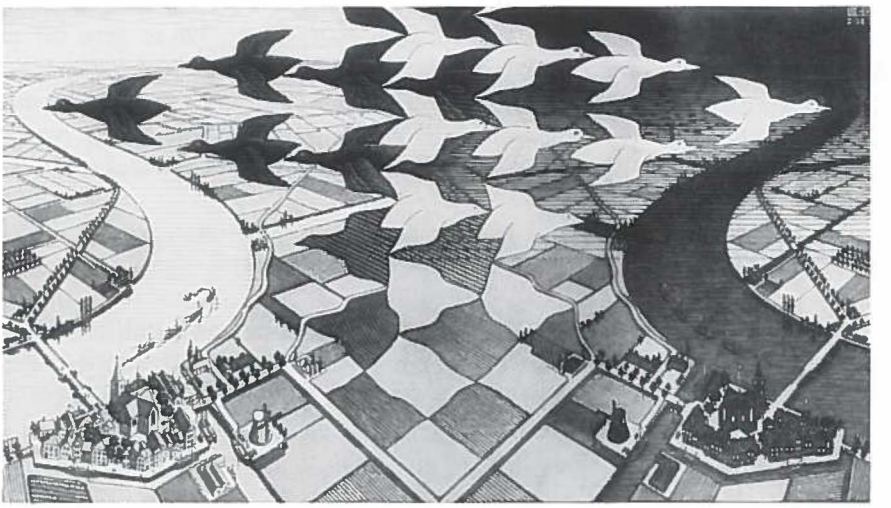
It is shown that the tumors of the patients who died of disease showed higher amounts of proliferative and mitotic activity *before* and *after* HILP as compared to those patients who were alive without disease. These findings suggest that patients with STS treated with HILP with a high proliferation index and high number of mitoses still have a worse prognosis, due to a known more aggressive behavior of high grade STS [8]. The HILP-TM generated changes in these tumor markers did not predict clinical outcome. With regard to the development of metastatic disease after HILP-TM it should be noted that in the patients with newly developed distant metastases, the number of mitotic cells was higher after HILP-TM as compared to the patients who did not develop distant metastases. Interestingly, this was not the case before HILP-TM, which implies that a high amount of dividing cells detected after HILP-TM could indicate patients who are at risk for the development of distant metastases. Although not significant, posttreatment proliferation was higher and posttreatment apoptosis was lower in the patients who developed new distant metastases after HILP-TM as compared to patients who did not. The importance of posttreatment proliferation index is also observed in the survival analysis in which a

low proliferative activity after HILP-TM is associated with a significantly better OS. Mitotic and apoptotic activity after HILP-TM is not clearly associated with survival.

In conclusion, the results of this study indicate that a better clinical response in HILP-TM is observed in patients with STS displaying high mitotic activity and low amounts of apoptosis. The antitumor effects of TNF-α and melphalan seem to result in a decrease in both mitotic activity and proliferation as well as an increase in apoptosis. Furthermore, patients with a favorable clinical outcome and survival have low proliferative STS both before and after HILP-TM, whereas patients who develop distant metastases after HILP-TM have relatively high numbers of dividing cells in the tumor remnants after treatment.

Chapter 6

PET WITH ^{18}FDG AND WITH L-1-[^{11}C]-TYROSINE IN RELATION TO THE HISTOPATHOLOGY OF SOFT TISSUE TUMORS BEFORE AND AFTER THERAPY



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SUMMARY

The current study was undertaken to investigate the relation of PET using FDG or L-1-[^{11}C]-tyrosine (TYR) with histopathological findings in soft tissue tumors, before and after therapy. Histopathological parameters that were studied were tumor grade, mitotic rate, proliferation activity and amount of necrosis.

PET with either FDG or TYR was performed in 55 patients with a lesion suspected to be a malignant soft tissue tumor. In twenty-eight patients a second PET study after therapy was performed. Metabolic rate of glucose consumption (MRglc) and protein synthesis rate (PSR) were calculated. Histological parameters were obtained from a biopsy specimen that was taken just after the first PET study and from the tumor remnant that was resected after therapy.

MRglc correlated with tumor grade (r=0.71) and mitotic rate (r=0.68), but not with proliferation or necrosis. After therapy there was no longer a correlation with mitotic rate. PSR correlated with tumor grade (r=0.53), mitotic rate (r=0.73) and proliferation (r=0.66). After therapy, correlation with mitosis and proliferation had improved, and a negative correlation was found between PSR and necrosis (r=-0.74).

These results validate the use of both FDG and TYR to give an in vivo indication of histological tumor parameters. However, FDG gives a better indication of tumor grade, whereas TYR is more accurate in predicting mitotic rate and proliferation, especially after therapy. FDG may therefore not be the most suited tracer for monitoring therapy. TYR might be more appropriate for that purpose.

INTRODUCTION

Although it seems obvious that a higher mitotic rate and proliferation require more energy, and that a loss of differentiation results in a less efficient and therefore higher metabolism, a clear correlation between metabolic and histologic parameters has not yet been demonstrated. With positron emission tomography (PET) the metabolism of tumors can be studied in vivo with the use of radiopharmaceuticals. The radiopharmaceutical ^{18}F-fluoro-2-deoxy-D-glucose (FDG) is the most widely used PET tracer in oncology. With FDG, the metabolic rate of glucose consumption (MRglc) can be visualized and quantified [259]. High glucose metabolism has been demonstrated with FDG-PET in several types of soft tissue tumors (STT) [21,171]. A relation between the glucose consumption and the malignancy grade has been shown, but very little is known about the correlation of glucose consumption with other histopathological tumor parameters.

Experimental studies have demonstrated that amino acid transport and protein synthesis rate (PSR) are increased in malignant cells [193,260]. The majority of the amino acid PET studies in oncology have been performed with L-[methyl-^{11}C]-methionine (MET), which is relatively easy to synthesize. In our institute L-1-[^{11}C]-tyrosine (TYR) is used for this purpose, because with TYR-PET the PSR can be quantified,

whereas MET-uptake reflects amino acid transport rather than PSR [196,261]. With TYR, several types of malignant tumors can be visualized [175]. The relation of PSR to histopathological tumor parameters has not been elucidated yet. The current study was undertaken to investigate the relation of both FDG-PET and TYR-PET with histopathological findings in STT, before and after therapy. STT comprise 1%of all malignant tumors. Patients with a STT have a poor prognosis (50% 5-year survival). The estimation of malignancy of STT depends on mitosis and proliferation. Mitotic activity and proliferation have prognostic value [29]. Histopathological parameters that were studied were tumor grade, amount of necrosis, mitotic rate and proliferation activity, as assessed with Ki-67 labeling.

PATIENTS AND METHODS

Patients

The FDG-PET group consisted of 30 patients, thirteen men and seventeen women, with a mean age of 50 (range 18-84) years (Table 1). The TYR-PET group consisted of 25 patients, nineteen men and six women, with a mean age of 55 (range 25-83) years (Table 2). All 55 patients were clinically and radiographically suspected of having a malignant STT, but histological examination after PET revealed 46 malignant and 9 benign STTs. Conventional imaging consisted of plain radiography, bone scintigraphy, computed tomography and/or MRI. All tumors were larger than 2 cm diameter. A biopsy to obtain a definite diagnosis was performed in all patients after the PET study, in order to avoid interference of wound healing on the PET signal. The study protocol was approved of by the medical ethics committee of the Groningen university hospital and all patients gave informed consent.

To evaluate treatment response, 28 patients underwent a second PET study (17 FDG-PET and 11 TYR-PET) after therapy, which consisted of hyperthermic isolated limb perfusion with tumor necrosis factor- and melphalan (HILP) as described previously [83].

PET studies

FDG was routinely produced by a robotic system following the procedure described by Hamacher, with a radiochemical purity of more than 98% [262]. TYR was produced via a modified microwave induced Bücherer-Strecker synthesis [263]. The radiochemical purity was over 99%. A 951/31 ECAT positron scanner (Siemens/CTI, Knoxville, USA) was used for data acquisition. The scanner acquires 31 contiguous tomographic slices simultaneously over a total axial length of 10.8 cm and has a spatial resolution of 6 mm at full width half maximum.

Patients fasted overnight prior to the PET study. Serum glucose levels (and in case TYR-PET was performed also tyrosine levels) were measured just before each PET session and were all within normal range. A 20 gauge needle was inserted into the radial artery under local anesthesia. An intravenous cannula was inserted into the contralateral cephalic vein for the injection of the tracer. The patients were positioned

supine in the scanner with the tumor in the field of view. A 20 minute transmission scan was obtained to correct for attenuation of the photons by the body tissues. Then 185 MBq FDG or 370 MBq TYR was intravenously administered and sequential images were acquired at the level of the lesion by obtaining 16 frames from the time of injection through 50 minutes postinjection. These include ten 30-seconds frames, three 5-minute frames, and three 10-minute frames. For establishing the input function, blood samples were taken simultaneously from the arterial cannula (at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2.25, 2.75, 3.75, 4.75, 7.5, 12.5, 17.5, 25, 35 and 45 minutes after injection). The same camera protocol was used for both tracers. The input function of the TYR-studies was corrected for ^{11}C-labeled metabolites.

PET data analysis

The MRglc and the PSR of the lesions were calculated as described in detail previously [174]. For calculating the MRglc or PSR the ten pixels with highest activity of the tumor were selected. When the lesion could not be visualized clearly, a region of interest was drawn around its location, based on MRI or CT-findings. After therapy, the metabolism was calculated over the same volume as before therapy to avoid observer-bias. Because it is known from a previous study that MRglc correlates better with malignancy grade than standardized uptake values, the latter were not calculated [21].

Histological examination

Histological parameters that were compared with the PET findings before therapy were obtained from a biopsy specimen that was taken after the first PET study. Those compared with the PET findings after therapy were obtained from the residual tumor that was resected as a whole, approximately eight weeks after HILP. Histological diagnosis was made on hematoxylin-eosin stained paraffin sections and additional immunohistological stains if necessary. All STT were classified according to Enzinger and Weiss [8] and graded according to the system of Coindre et al. [29]. Benign tumors were assigned tumor grade zero. The amount of necrosis was estimated on gross examination and microscopically. At least one section per centimeter of the largest tumor diameter was taken. The number of mitoses per 2 mm^2 (mitotiç index) was counted in fifteen adjacent fields on haematoxylin-eosin stained paraffin sections. Proliferation activity was assessed by Ki-67 labeling. Ki-67 is a nuclear antigen that is present during the whole cell cycle except for the G0 and G1 phase and is therefore a measure for proliferation. For Ki-67 labeling the monoclonal antibody MIB-1 (Immunotech S.A., Marseille, France) was used, which recognizes an epitope of the Ki-67 antigen. Ki-67 labeling was performed on paraffin sections (4 μm) according to a method modified from Shi et al. [177,178]. In short: after heating on a hot plate, slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After heating twice in an autoclave for 10 minutes at 110 °C in 20 mM citrate buffer (pH 6.0), slides were incubated with a 1:400 dilution of the antibody in maleate buffer (pH 7.4). The primary antibody was detected with a biotinylated secondary antibody followed by a streptavidine-alkaline phosphatase

conjugate (Ready-to-use link and label, Biogenex, San Ramon CA, USA). Final color was developed by the bromochlorindolyl-phosphate 4-nitroblue-tetrazoliumchloride method (Boehringer, Mannheim, Germany). For determining the Ki-67 labeling index (Ki-67 LI) we used ocular micrometry on a microscope with an eyepiece grid at x400 magnification. In histologically viable areas fifteen fields were randomly selected. The positive and negative nuclei were counted. Endothelial cells, inflammatory cells and necrosis were excluded. The Ki-67 LI was defined as the number of positive nuclei divided by the total number of nuclei in each of the fifteen fields. The mean Ki-67 LI of the fifteen fields was calculated.

Statistical analysis

To quantify the degree of correlation between the PET findings and the histological parameters a Spearman's (non-parametric parameters) or Pearson's correlation (parametric parameters) analysis was performed using SPSS statistical software. A p-value of <0.05 was considered to be significant. A correction for multiple comparisons was made by adjusting the p-level downward.

Table 1. Characteristics of patients that were studied with FDG-PET

	age & sex	histology	tumor grade
1	59, F	malignant fibrous histiocytoma	III
2	54, M	malignant fibrous histiocytoma	III
3	39, F	synovial sarcoma	III
4	40, F	leiomyosarcoma	III
5	84, F	sarcoma NOS	III
6	28, M	sarcoma NOS	III
7	53, M	malignant fibrous histiocytoma	II
8	80, M	malignant fibrous histiocytoma	II
9	39, F	malignant fibrous histiocytoma	II
10	65, F	malignant peripheral nerve sheath tumor	II
11	62, M	malignant peripheral nerve sheath tumor	II
12	43, M	malignant peripheral nerve sheath tumor	II
13	50, F	dedifferentiated liposarcoma	II
14	71, M	liposarcoma	II
15	18, F	rhabdomyosarcoma	II
16	56, F	primitive peripheral neuroectodermal tumor	II
17	18, M	extraskeletal myxoid chondrosarcoma	II
18	49, F	sarcoma NOS	II
19	64, M	sarcoma NOS	II
20	47, F	myxoid malignant fibrous histiocytoma	I
21	39, F	myxoid liposarcoma	I
22	44, F	myxoid liposarcoma	I
23	48, F	well differentiated liposarcoma	I
24	57, M	well differentiated liposarcoma	I
25	56, M	malignant fibrous histiocytoma	
26	38, M	myxoma	0
27	21, M	lymphangioma	0
28	43, F	lipoma	0
29	82, F	bursa	0
30	54, F	ganglion	0

NOS: not otherwise specified

RESULTS

In the FDG-PET group, PET was obtained in 29 patients before therapy (there was one technical failure). MRglc ranged from 0.6 to 99.1 μmol/100 g tissue/minute. There were six grade III tumors, twelve grade II, six grade I and five grade 0 (Table 1). One tumor was not graded, because there was too little biopsy material for accurate grading. Necrosis ranged from 0 to 70%, mitotic rate from 0 to 25 /2mm^2, and the Ki-67 LI ranged from 1.2 to 38.4. After therapy, MRglc (range 7.4-44.4), necrosis (range 0-100), mitotic rate (range 0-50), and Ki-67 LI (range 0-37.1) were obtained in seventeen patients.

Table 2. Characteristics of patients that were studied with TYR-PET

	Age & sex	histology	tumor grade
1	66, M	malignant fibrous histiocytoma	III
2	67, F	malignant fibrous histiocytoma	III
3	83, F	malignant fibrous histiocytoma	III
4	26, F	synovial sarcoma	III
5	45, M	leiomyosarcoma	III
6	71, F	fibrosarcoma	III
7	32, F	malignant peripheral nerve sheath tumor	III
8	67, M	pleiomorphic liposarcoma	III
9	75, M	sarcoma NOS	III
10	69, F	sarcoma NOS	III
11	76, M	sarcoma NOS	III
12	68, M	malignant fibrous histiocytoma	II
13	69, F	malignant fibrous histiocytoma	II
14	25, M	synovial sarcoma	II
15	49, M	myxoid liposarcoma	II
16	69, M	well differentiated liposarcoma	I
17	49, F	myxoid liposarcoma	I
18	58, M	myxoid liposarcoma	I
19	48, M	fibrosarcoma	I
20	43, M	clear cell sarcoma	I
21	51, M	malignant hemangiopericytoma	I
22	54, M	lipoma	0
23	42, M	lipoma	0
24	42, M	benign peripheral nerve sheath tumor	0
25	47, M	elastofibroma	0

NOS: not otherwise specified

Before therapy, a correlation was found between MRglc and tumor grade (r=0.71) and mitotic rate (r=0.68) (Figure 1a and 2a). There was no correlation between the MRglc and Ki-67 LI (Figure 3a) or amount of necrosis neither before nor after therapy (Figure 4a). After therapy, the correlation between MRglc and mitotic rate disappeared (r=0.18).

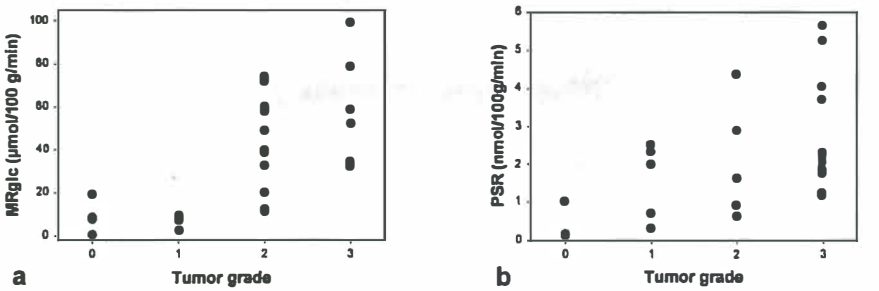


Figure 1
Metabolic rate of (a) glucose consumption (MRglc) and (b) protein synthesis rate (PSR) as measured with PET versus tumor grade. MRglc correlates better with tumor grade than PSR. Correlation-coefficients are 0.71 and 0.58 respectively.

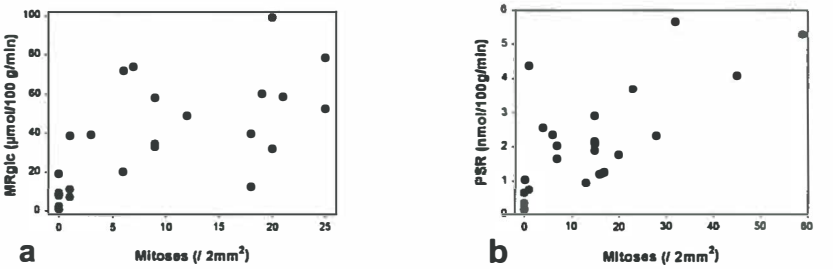


Figure 2
Metabolic rate of (a) glucose consumption (MRglc) and (b) protein synthesis rate (PSR) before therapy as measured with PET versus mitotic activity. Correlation-coefficients are 0.68 and 0.73, respectively.

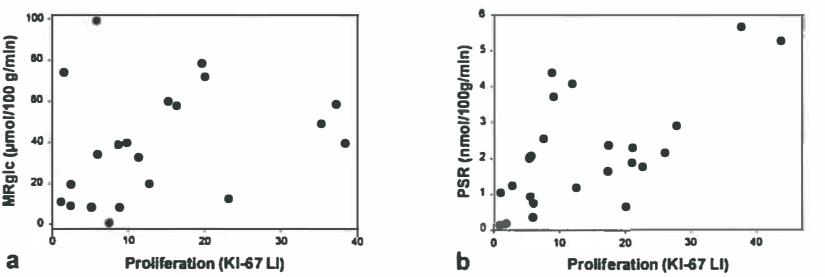


Figure 3
Metabolic rate of (a) glucose consumption (MRglc) and (b) protein synthesis rate (PSR) before therapy as measured with PET versus proliferation activity. The correlation between MRglc and the Ki-67 labeling index is not significant. The correlation-coefficient of PSR and proliferation is 0.66.

In the TYR-PET group, PET was obtained before therapy in 25 patients. PSR ranged from 0.13 to 5.66 nmol/kg tissue/minute. There were eleven grade III tumors, four grade II, six grade I and four grade 0 (Table 2). Necrosis ranged from 0 to 80%, mitotic rate from 0 to 59 /2mm^2, and the Ki-67 LI ranged from 0.9 to 43.8. After therapy, PSR (range 0.50-2.79), necrosis (range 0-59), mitotic rate (range 0-40) and the Ki-67 LI (range 0-33.0) were obtained in eleven patients.

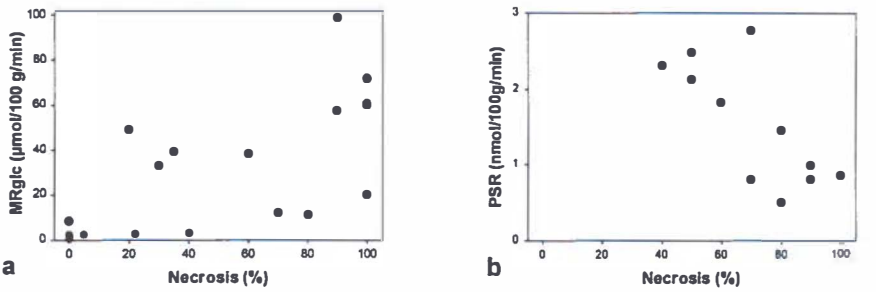


Figure 4
Metabolic rate of (a) glucose consumption (MRglc) and (b) protein synthesis rate (PSR) as measured with PET versus the amount of necrosis after therapy. There is no significant correlation between MRglc and the necrosis percentage, whereas there is a clear negative correlation between PSR and necrosis (r= -0.74).

Before therapy, a correlation was found between PSR and tumor grade (r=0.58) (Figure 1b), mitotic rate (r=0.73) (Figure 2b) and the Ki-67 LI (r=0.66) (Figure 3b). There was no correlation between the PSR and the amount of necrosis. After therapy, the correlation between PSR and mitotic rate (r=0.82), and between Ki-67 LI (r=0.87) had improved. There was now also a clear negative correlation between PSR and necrosis percentage (r=-0.74) (Figure 4b).

DISCUSSION

The current study demonstrates a clear correlation between both FDG-PET and TYR-PET on the one hand, and several histopathologic tumor parameters on the other. This correlation exists even though the tumor metabolic activity as measured with PET is compared to histopathological parameters from a biopsy specimen that may not be representative for the whole tumor.

In several previous PET studies with patients with STT, a relation was found between FDG uptake and malignancy grade [21,171]. Low grade tumors have lower FDG uptake than intermediate and high grade tumors. However, no significant difference was found between intermediate and high grade tumors. The present results may

elucidate this, because we did find a correlation between MRglc and mitotic rate, but not between MRglc and necrosis. Malignancy grade was determined by mitotic rate, differentiation and the amount of necrosis [29]. These parameters may have opposing effects on tumor metabolism as assessed with PET, especially in high grade tumors. A high mitotic rate requires more energy and results in a high cell density which in itself has been shown to correlate with FDG uptake in other tumor types [264,265]. Differentiation is based on the resemblance to the normal tissue and poor differentiation is associated with more aggressive growth and a tendency to metastasize. Necrosis is a clear sign of aggressive and poorly controlled growth and will thus be found more in high grade tumors with an increased rate of glucose consumption, but necrosis is not metabolically active. Therefore, with an increasing amount of necrosis, the average rate of glucose consumption of the tumor will decrease. Apparently, the same theory may be valid for TYR-PET. However, the influence of necrosis on the tumor PSR appears to be stronger than on MRglc, because the correlation coefficient of PSR and tumor grade is lower ($r=0.58$ versus $r=0.71$) and after therapy, when necrosis becomes more prominent, a clear negative correlation between PSR and necrosis is shown. The latter may be explained by the fact that inflammatory cells that are frequently seen in necrotic areas and the reactive inflammatory tissue in the tumor rim after therapy also have a high MRglc, but no high PSR [194,266-268].

FDG uptake was shown to be related to proliferation in patients with intracranial tumors, bronchial carcinoma and head-and-neck tumors [269-271]. However, we could not confirm this in patients with STT. From the results from Minn and Watanabe it was suggested that the increase in glucose utilization in head and neck cancers is mainly needed for nucleic acid synthesis [271,272]. However, larger changes in proliferation rate result in only moderate changes in FDG uptake [273]. In in vitro studies only a weak relation of FDG uptake to proliferative activity was established [264,274]. In patients with carcinoma of the hypopharynx, the relation between FDG accumulation and the proliferative index was evident ($r=0.80$), but only when the tumors with high and low FDG uptake were separated [275]. These data suggest that another factor contributes to the uptake of FDG in tumors. This unknown factor might be the expression of an oncogene, which enhances glucose transport or density of glucose transporter proteins, or a simultaneously occurring inflammatory reaction.

In studies with patients with lung, breast or head and neck cancer the uptake of the amino acid MET correlated well with the proliferative activity of the tumor [276-278]. In in vitro studies the relation between amino acid uptake and proliferation was better than with FDG [274]. We now validated these in vitro observations in the clinical situation in tumors with different growth characteristics using TYR-PET. In two studies of patients with various brain tumors, no correlation was found between PSR as measured with TYR-PET and proliferation [176,279]. Although brain tumors and soft tissue tumors are two entirely different entities, this suggests that there are also confounding factors influencing the PSR of tumors that are worth exploring.

Both MRglc and PSR correlated with mitotic rate. The disappearance of the correlation between MRglc and mitotic rate after therapy might be influenced by the

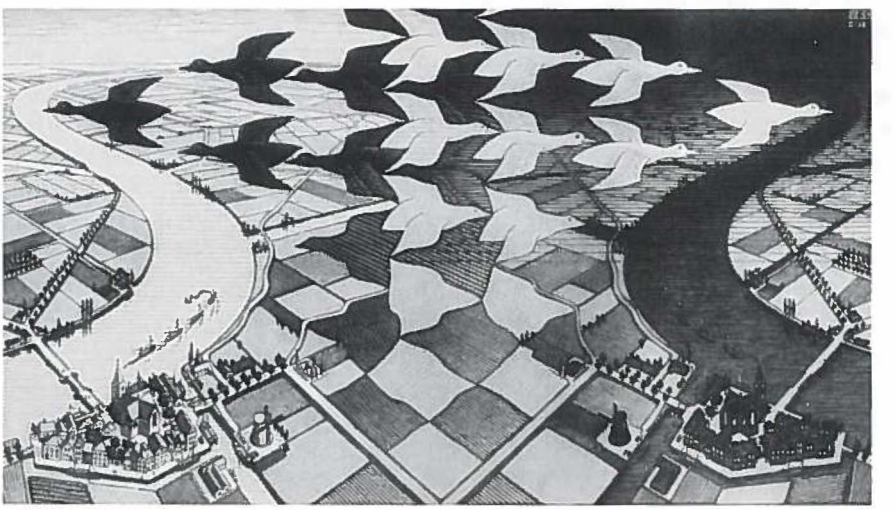
inflammatory reaction that was seen at all tumor sites after HILP. Except for tumor grade, TYR correlated better with all histological tumor parameters than FDG, especially after therapy. FDG may therefore not be the most suited tracer for monitoring therapy. This was also the conclusion in a previous study that studied the value of FDG-PET for monitoring treatment in similar patients [113]. TYR-PET may be more appropriate to monitor therapy. First results of TYR-PET for treatment evaluation that were reported recently, indeed confirmed less disturbance of inflammatory cells for TYR than for FDG, although neither with TYR a complete response could be distinguished from a partial response with 100% accuracy [210]. Further studies investigating the value of TYR-PET for monitoring therapy are therefore required.

In summary, the current study demonstrates the correlation of tumor grade and mitotic rate with MRglc as measured with FDG-PET, whereas there was no correlation with the amount of necrosis and proliferation. A correlation was found between the PSR as measured with TYR-PET on the one hand and tumor grade, mitotic rate and proliferation on the other hand. After therapy there was a clear negative correlation between the PSR and the amount of necrosis. FDG may not be the most suited tracer for monitoring therapy because of disturbing uptake by inflammatory cells. TYR-PET might be more appropriate for that purpose.

Acknowledgements
We gratefully acknowledge the financial support by the Dutch Cancer Society (Koningin Wilhelmina Fonds) grants RuG 94-786 and RUG 95-1085.

Chapter 7

EXPRESSION OF THE MULTIDRUG RESISTANCE ASSOCIATED PROTEINS P-gp, MRP_1 AND LRP IN SOFT TISSUE SARCOMAS



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SUMMARY

Responses to chemotherapy vary between specific types of soft tissue sarcomas (STS): poor responses are observed in leiomyosarcomas (LMS), whereas favorable responses are found in liposarcomas (LPS). A poor survival after chemotherapy for advanced STS is reported especially in malignant fibrous histiocytoma (MFH). High grade STS are associated with a favorable response to chemotherapy. Cross resistance to a number of functionally and structurally distinct natural product drugs is called multidrug resistance (MDR). MDR has been associated with the overexpression of P-glycoprotein (P-gp) and the multidrug resistance related protein-1 (MRP_1). Drug resistance is also associated with the expression of the lung resistance protein (LRP). The purpose of this study was to evaluate the expression of P-gp, MRP_1 and LRP with respect to histological type and tumor grade in a series of 115 STS.

MDR-protein expression was semiquantitatively assessed after immunohistochemical detection in paraffin embedded tumor material of 115 chemotherapy naive STS (23% grade I, 47% grade II and 30% grade III).

At least one MDR protein was detected in 95% of the STS. Co-expression of P-gp and MRP_1 was found in 32%, P-gp and LRP in 48% and MRP_1 and LRP in 28% of the analyzed STS. Expression of all 3 MDR proteins was found in 21%. Expression of P-gp (71% of the STS) and LRP (70% of the STS) was more observed than expression of MRP_1 (36% of the STS) ($p<0.01$). P-gp expression was not correlated to either MRP_1 or LRP and MRP_1 expression was not correlated to LRP expression. P-gp expression was most pronouced in MFH and synovial sarcomas and low in LMS. MRP_1 was most expressed in malignant peripheral nerve sheath tumors and low in rhabdomyosarcomas. LRP was most expressed in MFH and sarcoma not otherwise specified and low in LPS. All MFHs expressed at least one MDR protein. No correlation was found between tumor grade and the expresssion of P-gp, MRP_1 or LRP. Co-expression was less frequently observed in grade I STS.

In conclusion, P-gp, MRP_1 and LRP are expressed in the majority of STS, whereas co-expression is frequently found. Expression of P-gp, MRP_1 and LRP varies between histological types and is not related to tumor grade. Co-expression is associated with low grade STS.

INTRODUCTION

Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumors which arise from tissue of mesenchymal origin and account for approximately 1% of all malignancies. Approximately 30% of the patients with STS will develop distant metastases, predominantly in the lungs [8,9]. Chemotherapy is the treatment of choice in patients with diffuse distant metastases, but is also applied in advanced inoperable sarcomas and childhood rhabdomyosarcomas [86,280]. Doxorubicin, alone or in combination with other cytostatic drugs such as ifosfamide or etoposide, is the most

extensively applied cytotoxic drug in adult STS patients and can be regarded as the standard chemotherapeutic agent in STS [86,87,281-284]. Response rates in general are 20-30%, although response rates of up to 57% have been reported, and 2 years overall survival is 22% [87]. Recent clinical studies indicated that the response to chemotherapy is related to histological grade and the histological subtype, e.g. leiomyosarcomas have poor responses and liposarcomas have favorable responses [87,142,285,286]. Patients with metastasized malignant fibrous histiocytomas (MFH) have a worse survival time after chemotherapy than other histological types [87]. Poor responses to chemotherapy may be caused by so called multidrug resistance (MDR).

MDR can be caused by one or more mechanisms, i.e. 1) increased expression of energy dependent efflux pumps P-glycoprotein (P-gp) or the multidrug resistance related protein-1 (MRP_1) as well as 2) an increased expression of the lung resistance related protein (LRP) and the multidrug resistance related protein-1 (MRP_1) [96,97,287-295]. A third possible mechanism of MDR is 3) a reduced expression of topoisomerase II. P-gp is localized on the cell membrane, MRP_1 is localized both cytoplasmic and on the cell membrane. The spectrum of resistance for cytostatic agents which are involved in MDR (such as the anthracyclines, the vinca-alkaloids and epipodophyllotoxins) does not differ much between P-gp and MRP_1. LRP, which was identified as the human major vault protein is located intracytoplasmic and has been considered to play a role in the transport between nucleus and cytoplasm. Until now, the role of LRP in MDR is less clear, although some studies in ovarian cancer, acute myeloid leukemia and multiple myeloma indicated a possible role in clinical drug resistance [96,97,295,296].

P-gp expression has been detected in STS and might be associated with poor response to chemotherapy in childhood rhabdomyosarcomas as well as adult STS [100-103]. MRP_1 has been detected in STS and co-expression of MRP_1 and P-gp was associated with tumor grade in STS [108,297]. The combined expression of P-gp, MRP_1 and LRP in the various histological types of STS is unknown. The purpose of this study was to evaluate the expression of P-gp, MRP_1 and LRP with respect to histological type, especially LMS, LPS and MFH, and tumor grade in a series of 115 STS.

MATERIALS AND METHODS

Histology

The criteria for inclusion in the current study were 1) an untreated primary tumor, 2) a histological diagnosis of a STS and 3) the availability of paraffin embedded tumor tissue. All tumors were randomly selected from a STS database. However, a relatively high proportion of leiomyosarcomas and liposarcomas was selected since these groups were specifically reported in previous clinical studies responses [87,142,285,286].

The studied group consisted of 115 STS, obtained from 54 male and 61 female patients (mean age: 49, median: 48, SD: 17.9, range: 16-89 years). In all cases the histological diagnosis was made on haematoxylin-eosin stained paraffin

sections with or without additional immunohistological stains. All cases were classified according to Enzinger and Weiss [8] which revealed 26 (23%) leiomyosarcomas (LMS), 24 (21%) liposarcomas (LPS), of which were 9 of the myxoid subtype, 13 (11%) malignant fibrous histiocytomas (MFH), 12 (10%) sarcomas not otherwise specified (NOS), 11 (10%) rhabdomyosarcomas (RMS), 9 (8%) synovial sarcomas, 7 (6%) malignant peripheral nerve sheath tumors (MPNST), 3 (2%) fibrosarcomas and 10 (9%) other STS. The STS were graded according to the grading system of Coindre et al. [29], which resulted in 26 grade I (23%), 54 grade II (47%) and 35 grade III (30%) STS.

MDR expression

For detection of P-gp, the monoclonal antibody C494 (Signet Laboratories, Dedham MA, USA) in a concentration of 120 µg/ml in phosphate-buffered saline (PBS) (Merck KgaA, Darmstadt, Germany) plus 1% bovine serum albumin (BSA) (Serva Electrophoresis GmbH, Hamburg, Germany) was used. For detection of MRP_1 the monoclonal antibody MRPr1 (provided by Dr. R.J.Scheper, Dept. of Pathology, Free University Hospital, Amsterdam) in a concentration of 20 µg/ml in 1% BSA/PBS was utilized. For detection of LRP the monoclonal antibody LRP (Transduction Laboratories, Los Angeles CA, USA) in a concentration of 250 µg/ml in 1% BSA/PBS was used. As controls for immunohistochemal detection served cytospins of the well documented cell lines A2780 and GLC4 and their corresponding multi-drug resistant sublines, i.e. overexpressing P-gp (A2780 AD), MRP_1 (GLC4/ADR) and LRP (GLC4/ADR) [298,299], as well as paraffin embedded formalin fixed liver (P-gp), lung (MRP_1) and colon (LRP) tissue.

Paraffin sections (3 µm) were placed on positively charged glass slides and were air dried. Immunohistochemistry was performed according to a method modified from Shi et al. [177,178] . Briefly, after heating on a hotplate, the slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After each of the subsequent steps, three 5 minutes washes in PBS were carried out. After three times heating in an autoclave for 5 minutes at 115° C in a 20 mM blocking reagens (Boehringer Mannheim, Mannheim, Germany) with pH=6.0, endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in PBS during 30 minutes. The slides were incubated with the specific antibody in 1% BSA/PBS (pH=7.4) for 1 hour at room temperature in a humidified chamber. The primary antibody was detected with a rabbit antimouse (DAKO, Glostrup, Denmark) (C494 and LRP) or rabbit antirat (DAKO, Glostrup, Denmark) (MRPr1) peroxidase labeled secondary antibody diluted in 1:50 + 1% human serum (type AB) followed by incubation with goat antirabbit conjugated peroxidase diluted in 1:50 +1% human serum (type AB). 3.3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA) with imidazole (Merck KgaA, Darmstadt, Germany) in PBS was used as the chromagen according to the manufacturer's instructions. After counterstaining with Mayer's haematoxylin, the slides were dehydrated through graded alcohols and mounted with coverslips.

The expression of P-gp, MRP_1 and LRP was assessed independently by two observers (B.P, H.H.) without knowledge of the histological diagnosis or clinical data. The P-gp, MRP_1, LRP proteins were studied in adjacent slides of the most viable parts of the tumor. Amount of P-gp, MRP_1 and LRP expression was semiquantitatively assessed by estimating the percentage of positive stained tumor cells and assessing staining intensity using a scale of 0 -3 (0: no staining, 1: very weak staining, 2: intermediate staining, 3: strong staining). Strong staining intensity was comparable to the staining intensity of the simultaneously stained positive control tissue. Both staining parameters were also combined into a so called histopath-score which is defined as: the estimated percentage of stained cells (0-100%) x the staining intensity (0-3) [300]. According previous studies, tumor samples were considered negative for expression of each of the proteins if ≤ 5% of the cells were positive [96,103]. It was not possible to determine P-gp expression in 3 cases and MRP_1 expression in 5 cases due to insufficient paraffin embedded tumor material.

Statistics

A Mann Whitney Test was carried out to analyze the differences in MDR parameters. To avoid the problem of multi-comparison in the analyses of P-gp, MRP_1 and LRP, a Bonferroni correction factor of 3 was introduced and in the comparison of the different histological types the Dunn's multiple comparison test was used. To quantitate the degree of correlation between parameters, the Spearman's rank test was used. A two-tailed p-value of < 0.05 was considered to be significant. Statistical software SPSS 9.0 for Windows (SPSS Inc., Chicago IL, USA) was used for statistical analysis.

RESULTS

MDR protein expression in STS

P-gp expression was found in 80 of the 112 (71%) analyzed tumors. There were 43 MRP_1 positive (36%) and 80 LRP positive (70%) STS. At least one MDR protein was detected in 101/107 (94%) of the analyzed STS. As shown in Tables 1-3, P-gp and LRP expression in this series of STS was each significantly higher than the MRP_1 expression ($p<0.01$). The amount of P-gp expression was not correlated with either MRP_1 or LRP expression, whereas the amount of MRP_1 expression was not correlated with LRP expression.

Co-expression of P-gp and MRP_1 was found in 34/107 (32%) STS, co-expression of P-gp and LRP in 54/112 (48%) and co-expression of MRP_1 and LRP in 31/110 (28%) of the analyzed STS. Expression of all three MDR proteins was found in 22/107 (21%) of the STS (Table 4). Expression of P-gp and MRP_1 was observed in both the cytoplasm and on the cell membrane, whereas LRP staining was found exclusively in the cytoplasm (Figure 1).

Table 1. P-glycoprotein (P-gp) expression in the various histological subtypes.

Diagnosis	all STS	FIBR	MFH	LPS	LMS	RMS	SYNS	MPNST	S-NOS	other STS
number of tumors	112	3	13	24	25	10	9	7	11	10
positive tumors:	71%	100%	100%	83%	32%	70%	89%	71%	64%	90%
positive cells:										
mean (%):	42	37	65	51	13	50	56	43	31	59
median (%):	40	30	70	55	5	55	80	50	30	70
SD (%):	34.0	11.5	19.8	30.2	23.0	38.0	39.6	34.5	33.8	32.5
range (%):	0-100	30-50	40-90	0-90	0-80	0-100	0-95	0-90	0-90	0-90
[a]histopathscore:										
mean:	74	63	112	86	22	114	78	76	42	117
median:	60	60	70	75	10	120	80	60	30	80
SD:	75.6	35.1	91.9	65.1	38.5	95.8	65.3	93.2	41.9	92.2
range:	0-300	30-100	40-270	0-240	0-160	0-300	0-190	0-270	0-100	0-270

STS: soft tissue sarcoma; FIBR: fibrosarcoma, MFH: malignant fibrous histiocytoma;
LPS: liposarcoma; LMS: leiomyosarcoma; SYNS: synovial sarcoma; MPNST: malignant peripheral nerve sheath tumor; S-NOS: sarcoma not otherwise specified
[a]histopath score: % positive cells x staining intensity (0-3)

Table 2. Multidrug resistance protein (MRP_1) expression in the various histological subtypes.

Diagnosis	all STS	FIBR	MFH	LPS	LMS	RMS	SYNS	MPNST	S-NOS	other STS
number of tumors	110	3	10	24	24	11	9	7	12	10
positive tumors:	39%	33%	40%	33%	33%	18%	33%	71%	42%	70%
positive cells:										
mean (%):	18	23	19	15	13	2	22	25	27	32
median (%):	1	0	0	3	0	0	0	20	1	40
SD (%):	27.4	40.4	31.1	27.7	22.9	4.0	34.8	24.3	34.9	26.7
range (%):	0-90	0-70	0-80	0-90	0-80	0-10	0-90	0-70	0-90	0-80
[a]histopath score:										
mean:	74	23	27	32	18	5	25	40	63	61
median:	60	0	0	4	0	0	0	30	1	40
SD:	75.6	40.4	47.2	68.1	33.0	10.3	37.1	41.6	89.0	75.4
range:	0-300	0-70	0-140	0-270	0-140	0-30	0-90	0-120	0-270	0-240

STS: soft tissue sarcoma; FIBR: fibrosarcoma, MFH: malignant fibrous histiocytoma;
LPS: liposarcoma; LMS: leiomyosarcoma; SYNS: synovial sarcoma; MPNST: malignant peripheral nerve sheath tumor; S-NOS: sarcoma not otherwise specified
[a]histopath score: % positive cells x staining intensity (0-3)

Table 3. Lung resistance protein (LRP) expression in the various histological subtypes.

Diagnosis	all STS	FIBR	MFH	LPS	LMS	RMS	SYNS	MPNST	S-NOS	other STS
number of tumors	115	3	13	24	26	11	9	7	12	10
positive tumors:	70%	33%	92%	42%	88%	54%	55%	86%	92%	60%
positive cells:										
mean (%):	42	28	59	23	58	32	31	21	62	43
median (%):	40	5	60	5	60	10	10	20	75	45
SD (%):	34.3	44.8	18.0	32.2	27.3	40.3	38.9	15.4	28.9	39.4
range (%):	0-90	0-80	30-90	0-90	0-90	0-90	0-90	5-50	5-90	0-90
[a]histopath score:										
mean:	85	57	125	41	111	81	71	42	110	96
median:	60	10	120	8	90	10	10	20	85	55
SD:	83.6	89.6	55.6	67.0	73.6	109.7	98.7	50.8	87.0	103.8
range:	0-270	0-160	60-270	0-240	0-270	0-270	0-270	5-150	5-270	0-270

STS: soft tissue sarcoma; FIBR: fibrosarcoma, MFH: malignant fibrous histiocytoma;
LPS: liposarcoma; LMS: leiomyosarcoma; SYNS: synovial sarcoma; MPNST: malignant peripheral nerve sheath tumor; S-NOS: sarcoma not otherwise specified
[a]histopath score: % positive cells x staining intensity (0-3)

Table 4. Percentage of tumors with co-expression of the MDR proteins in the analyzed histological subtypes.

Diagnosis	all evaluable STS	FIBR	MFH	LPS	LMS	RMS	SYNS	MPNST	S-NOS	other STS
no MDR protein	6%	0%	0%	8%	9%	10%	11%	0%	0%	0%
P-gp and MRP1	32%	33%	40%	29%	13%	20%	33%	57%	36%	60%
P-gp and LRP	48%	33%	92%	33%	32%	40%	56%	57%	55%	50%
MRP$_1$ and LRP	28%	0%	40%	17%	33%	9%	11%	57%	42%	40%
all 3 MDR proteins	21%	0%	40%	13%	13%	10%	11%	43%	37%	30%

MDR: multdrug resistance; P-gp: P-glycoprotein, MRP$_1$: multidrug resistance protein, LRP: lung resistance protein; STS: soft tissue sarcoma; FIBR: fibrosarcoma, MFH: malignant fibrous histiocytoma;
LPS: liposarcoma; LMS: leiomyosarcoma; SYNS: synovial sarcoma; MPNST: malignant peripheral nerve sheath tumor; S-NOS: sarcoma not otherwise specified
[a]histopath score: % positive cells x staining intensity (0-3)

MDR protein expression in relation to histology

The mean percentages of P-gp, MRP$_1$ and LRP positive cells for the various histological types has been depicted in Figure 2. The combined expression of MDR proteins was high in both MFH and sarcomas NOS. P-gp expression was most pronouced in MFH and synovial sarcomas, although the staining intensity was not as high as in MFH (Table 1). P-gp expression was low in LMS. MRP$_1$ expression was most pronounced in MPNST, while it was extremely low in RMS and LMS (Table 2). LRP was most expressed in MFH and sarcoma NOS, while it was low in LPS and LMS (Table 3). Co-expression of P-gp and LRP was frequently detected and all MFHs expressed at least one MDR protein (Table 4). The expression of P-gp, MRP$_1$ and LRP in MFH, LPS and LMS is shown in Table 5.

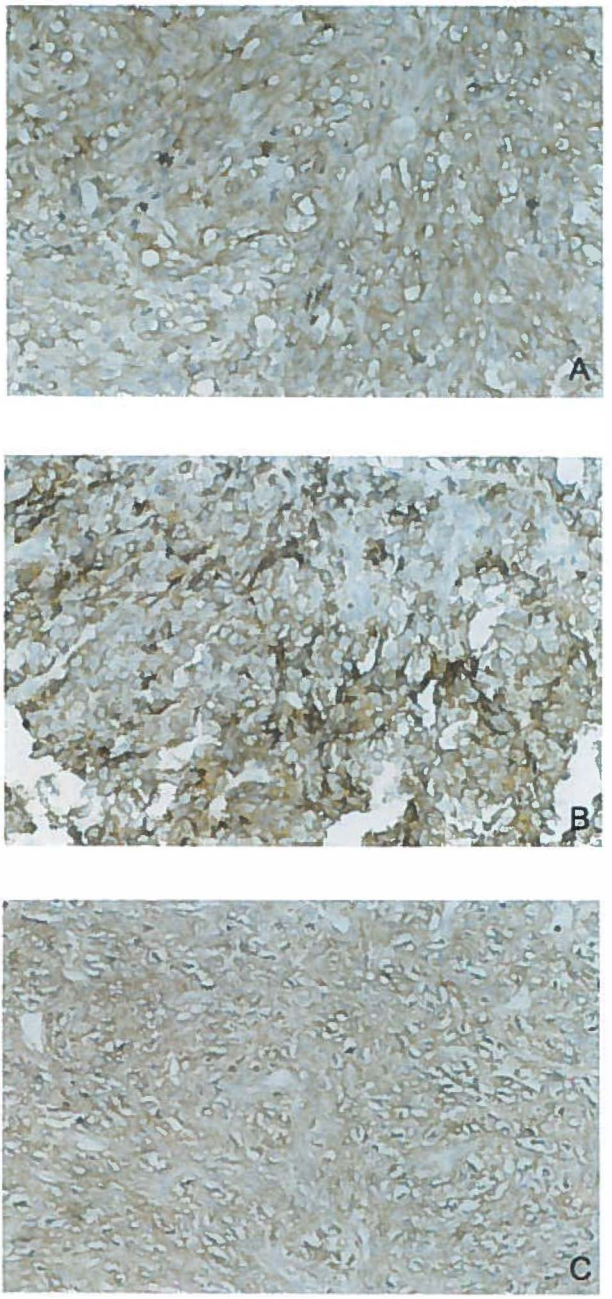


Figure 1.
Examples of MDR-protein expression in STS: (**A**) P-gp expression in an epthelioid sarcoma (200x), (**B**) MRP_1 expression in a rhabdomyosarcoma (200x) and (**C**) LRP expression in a malignant hemangiopericytoma (200x)

Table 5. Median MDR protein expression in MFH, LPS and LMS.

Diagnosis	MFH grade II	MFH grade III	well diff LPS	other LPS	myxoid LPS	LMS grade I	LMS grade II	LMS grade III
number of tumors	7	6	6	9	9	8	13	5
positive cells:								
P-gp (%):	70	65	45	80	40	3	5	5
MRP_1 (%):	0	10	8	5	0	0	20	0
LRP (%):	60	60	15	50	0	65	70	40
°histopath score:								
P-gp:	70	65	60	70	100	4	10	5
MRP_1:	0	20	13	5	0	0	20	0
LRP:	120	130	20	90	0	115	90	80

MDR: multidrug resistance; P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein; MFH: malignant fibrous histiocytoma; LPS: liposarcoma; LMS: leiomyosarcoma; °histopath score: % positive cells x staining intensity (0-3)

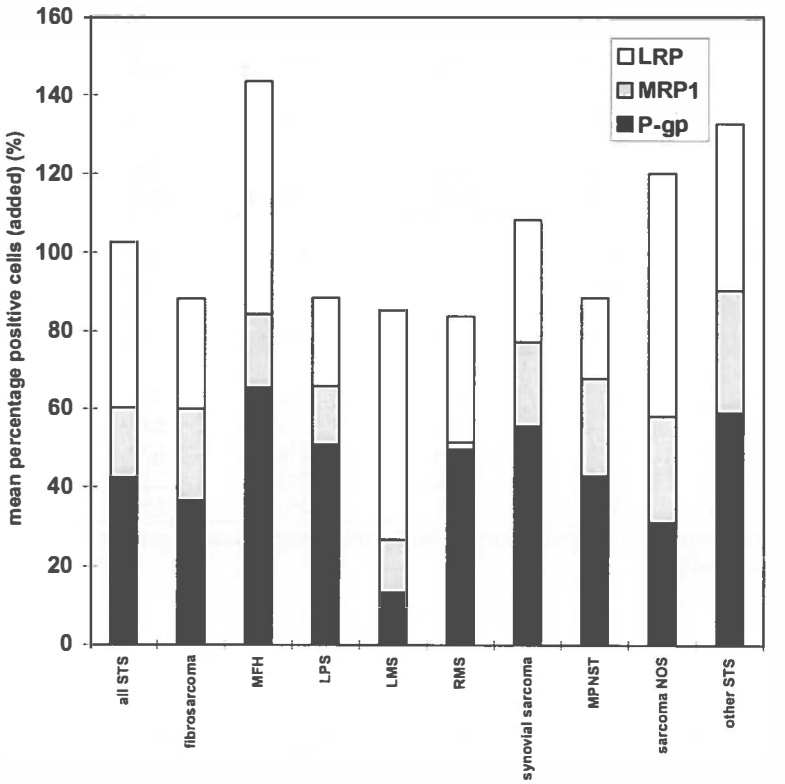


Figure 2.
Schematic overview of the mean expression of P-gp, MRP_1 and LRP in the various histological types.

MDR protein expression in relation to tumor grade

Expression of the evaluated MDR proteins in relation to tumor grade is shown in Table 6. No correlation was found between tumor grade and the expresssion of either P-gp, MRP_1 or LRP. However, co-expression of two or all three MDR proteins was less frequently observed in grade I (26%) and more in grade III (72%) STS ($p<0.001$). Higher tumor grade was associated with co-expression of P-gp and LRP, since 4/26 (15%) grade I, 28/51 (55%) grade II and 22/35 (63 %) of the STS co-expressed P-gp and LRP ($p<0.001$). No association existed between tumor grade and the co-expression of either P-gp and MRP_1 or MRP_1 and LRP.

Table 6. P-gp, MRP1 and LRP expression in relation to tumor grade.

	all STS	grade I	grade II	grade III
Number of tumors	115	26	54	35
P-gp positive cells:				
mean (%):	42	36	42	48
median (%):	40	25	40	40
SD (%):	34.0	34.3	33.9	33.8
Range (%):	0-100	0-90	0-95	0-100
MRP_1 positive cells:				
mean (%):	18	13	20	18
median (%):	1	0	5	0
SD (%):	27.4	24.0	27.1	30.6
Range (%):	0-90	0-90	0-90	0-90
LRP positive cells:				
mean (%):	42	27	48	45
median (%):	40	8	50	40
SD (%):	34.3	33.8	33.1	33.9
Range (%):	0-90	0-90	0-90	0-90
Co-expression:				
No MDR protein (%)	6%	4%	6%	6%
P-gp and MRP_1 (%)	32%	23%	35%	34%
P-gp and LRP (%)	48%	15%	55%	60%
MRP_1 and LRP (%)	28%	19%	37%	22%
all 3 MDR proteins (%)	21%	12%	24%	22%

MDR: multidrug resistance; P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein

DISCUSSION

The present study evaluates the expression of P-gp, MRP_1 and LRP in a group of 115 STS. It was found that (co-)expression is frequently observed in STS, although differences exist between the various histological types. Co-expression of MDR proteins is associated with higher tumor grade.

P-gp expression in this study was found in 71% of the analyzed STS. In several previous studies in STS the percentage of P-gp positive STS ranged between 18% and 62% [100,101,104-106,301,302]. However, differences in tissue preparation and

storage as well as the use of different techniques and monoclonal antibodies might explain the diverse results in relatively small groups of STS [303]. Oda et al. [297] found that only 47% of the MDR1 mRNA positive tumors expressed P-gp as determined by immunohistological detection. Furthermore, it appears that not all immunistologically detected P-gp expressing tumors have functional P-gp [304]. The discrepancy between the amount of RT-PCR detected MDR1 mRNA, the immunohistologically detected P-gp expression and the functional P-gp efflux pump, illustrates the problem of interpreting the few studies on P-gp expression in STS. P-gp expression using the C494 antibody was found both in the cytoplasm and in the cell membrane, as has been described earlier [103]. Although a correlation between P-gp expression and tumor grade was observed in some studies, it could not be confirmed in the present study [100,297]. Nakanishi et al., reported a relation between tumor grade and P-gp expression when intermediate and high grade tumors were compared with low grade STS. However, a relatively high proportion of MFH (24/55), most of which are intermediate or high grade, were included in their study [100]. This is confirmed in the present study, since all MFH were P-gp positive and had the highest amount of P-gp expressing cells when compared with the other subtypes. Thus the reported [100] relation between tumor grade and P-gp expression could also depend on the studied histological type.

Much less is known about MRP_1 expression in STS. In our group MRP_1 expression was detected in 36% of the STS and co-expression with P-gp was observed in 32% of the cases. Oda et al. used RT-PCR for the detection of MRP_1 mRNA and found co-expression with the MDR1 mRNA in 38% [108]. MRP_1 staining was found both in the cytoplasm and in the cell membrane as has been observed previously [305]. The present study showed that MRP_1 expression in STS is less pronounced than P-gp and LRP expression. Further studies should determine the clinical significance of the low MRP_1 expression and investigate whether the expression of MRP_1 is influenced by the high expression of P-gp, LRP or other ATP binding cassette proteins involved in MDR such as MRP_2 or MRP_6. In the earlier mentioned study of Oda et al. , a correlation of tumor grade with co-expression of MDR1 and MRP_1 mRNA was found. The relation between tumor grade and P-gp-MRP_1 co-expression could not be confirmed in the present study, which might be explained by the differences in the utilized techniques.

No reports, so far, have been published on the expression of LRP in STS. In this study it was found that 70% of the STS was LRP positive. LRP expression was predominantly found in the cytoplasm, as has been described earlier [96,292]. Expression of LRP was not correlated with P-gp or MRP_1 . This might indicate that expression of LRP is regulated by other factors than the membrane efflux pumps P-gp and MRP_1. The exact role of LRP in drug resistance is as yet unclear and it is thought that LRP is also involved in the transport of other cytotoxic agents, in particular alkalyting agents such as ifosfamide, than the drugs transported by P-gp or MRP_1 [289,296,306]. However, 48% of the STS were both LRP and P-gp positive and 28% of the STS showed co-expression of MRP_1 and LRP. This indicates that chemotherapy in STS should contain several compounds to overcome the different resistance

mechanisms on tumor level. It is expected that co-expression is also present on cellular level, since many of the analyzed tumors had LRP and P-gp expression in more than 50% of their cells. Future studies using double-staining techniques could further elucidate the co-expression on a cellular level. The co-expression of LRP and P-gp or the co-expression of two or three MDR proteins, regardless which two or three proteins were expressed, was associated with higher tumor grade.

Expression of P-gp, MRP_1 and LRP varies between and within the different histological types and no clear pattern can be discovered. MFH and sarcomas NOS seem to be characterized by high levels of MDR expression, predominantly P-gp and LRP in MFH which might explain the reported poor survival after adriamycin based chemotherapy [87]. LPS, which have favorable responses to doxorubicin based chemotherapy, are characterized by relatively low expression of MDR proteins. However, when is focussed on the different histological subtypes of LPS it should be noticed that myxoid LPS have almost no LRP expression and that MRP_1 expression is moderate. This could be explained by the influence of the chromosomal aberrations i.e. the t(12;16)(q13,p11) characteristic for myxoid LPS: the breakpoint 16p11.2 might affect expression of the LRP gene on 16p11.2 and the MRP_1 gene on 16p13. The other LPS have higher MDR expression. However, well differentiated LPS do not metastasize and are therefore probably not included in the study of Van Glabbeke et al., whereas the other non-myxoid LPS are not frequently diagnosed [8,87]. A high expression of P-gp in MFH, LPS and synovial sarcoma, observed in previous studies, was confirmed in our study, but the reported high expression of P-gp in LMS could was not observed in the present study [100,106,297]. The histological diagnosis of LMS and their resistance to chemotherapy is the subject of debate during the last years. In many previous studies on leiomyosarcomas, a large proportion of the tumors was derived from the digestive tract. There is now growing evidence that these tumors are derived from the cells of Cajal and are diagnosed as gastrointestinal stromal tumors (GIST). They the have to be distinguished from LMS, both histopathologically and clinically [307-310]. A recent study concluded that P-gp and MRP_1 expression in LMS is lower than in GIST and the reported drug resistance in LMS has to be interpreted with caution [87,311].

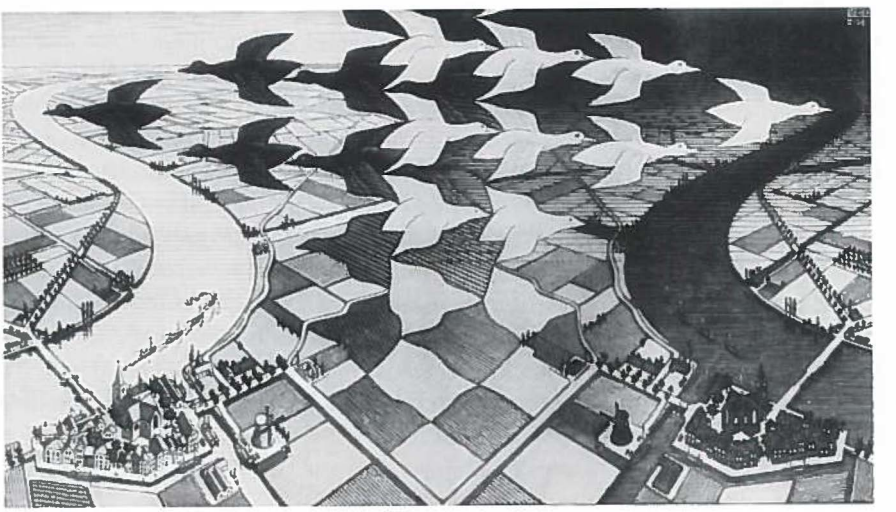
RMS had a relative absence of MDR co-expression when compared with the other STS: in 10% of the cases no MDR expression was found at all and in only 10% of the cases all 3 MDR proteins were expressed. RMS are found in relatively young patients and have a better response to vinca-alkaloids containing chemotherapy [312]. In the current study, the RMS patients (mean age: 26 years) were younger than the other STS patients ($p<0.01$). This implies that there might be a relation between the expression of MDR, histology and the age of the patient. When the correlation of the MDR parameters was analyzed, a weak correlation was found between age and the expression of both P-gp (r: -0.21, p=0.03) and LRP (r: 0.30; 0=0.001). This indicates that P-gp expression in STS is more frequently found in younger patients and that LRP expression increases with age. Further studies have to investigate the relation between patient age, histological type, drug resistance, chemotherapeutic response and clinical outcome.

STS have a relatively high expression of all three MDR proteins. In breast cancer, another doxorubicin treated solid tumor, P-gp expression was observed in 10 - 50% of the cases, MRP_1 in 80% and LRP in 75%[313]. In ovarian cancer, P-gp was detected in 16-17% of the tumors, MRP_1 in 44-68% and LRP in 74-77% [96,314].

In conclusion, 94% of the STS express either P-gp, MRP_1 or LRP and co-expression of two or three MDR proteins is found in 61%, which might explain the relative poor responses to polychemotherapy for STS. This studies shows that MDR expression varies between and within the histological subtypes and is most pronounced in MFH and sarcoma NOS. Co-expression seems to be associated with high tumor grade.

Chapter 8

SOFT TISSUE LEIOMYOSARCOMAS AND MALIGNANT GASTROINTESTINAL STROMAL TUMORS: DIFFERENCES IN CLINICAL OUTCOME AND IN EXPRESSION OF MULTIDRUG RESISTANCE PROTEINS



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SUMMARY

Several studies have reported clinical behavior and drug resistance in leiomyosarcomas, but did not differentiate between soft tissue leiomyosarcomas (LMS) and malignant gastrointestinal stromal tumors (GIST). Multidrug resistance (MDR) has been associated with the expression of P-gp (P-glycoprotein), MRP_1 (multidrug resistance protein) and LRP (lung resistance protein). The aim of the present study was to compare LMS and GIST with respect to clinical outcome and MDR parameters.

Of 29 previously untreated patients with a primary deep seated LMS and 26 previously untreated patients with a primary malignant GIST, clinical outcome was evaluated. Paraffin embedded material, available in 26 LMS and 25 GIST cases, was used for immunohistochemical detection of P-gp, MRP_1 and LRP.

The mean OS in patients with a LMS was 72 months and 31 months in patients with a GIST ($p<0.05$). Metastases occurred in 15 (58%) of 26 evaluable LMS patients and in 9 (53%) of 17 evaluable GIST patients. The lungs were the primary affected metastatic site in 13 (87%) of the metastasized LMS and in one of the GIST patients ($P<0.001$), while subsequent liver metastases developed in 4 LMS patients. In GIST patients with known metastatic disease during follow-up, liver metastases were found in 5 patients and intra-abdominal metastases in 4 patients. P-gp and MRP_1 expression was significantly more pronounced in GIST than in LMS ($p<0.05$). Mean percentage of P-gp expressing cells in LMS was 13.4%, while it was 38.4% in GIST. Mean percentage MRP_1 positive cells was 13.3% in LMS and 35.4% in GIST. LRP expression did not differ between LMS and GIST. In LMS, OS tended to be related to P-gp status: 88 months (P-gp-) versus 33 months (P-gp+), but not to MRP_1 or LRP status. In GIST, OS was not related to MDR status.

LMS patients have a better survival than GIST patients. LMS predominantly metastasize to the lungs, while GIST tend to spread to the liver and the abdominal cavity. LMS have a less pronounced drug resistance pattern than GIST. Future studies evaluating chemotherapeutic regimens should therefore differentiate between LMS and malignant GIST.

INTRODUCTION

Leiomyosarcomas (LMS) are a group of malignant tumors which arise from tissue of mesenchymal origin and have histologic features consistent with smooth muscle differentiation. They account for approximately 10% of all soft tissue sarcomas .[8,9] However, it appears that the tumors with smooth muscle differentiation arising in the digestive tract have to be considered as a distinct tumor entity derived from the interstitial cells of Cajal, although the histogenesis of these malignant gastrointestinal stromal tumors (GIST) is still subject of debate .[307-310,315-319] In several studies, metastatic "leiomyosarcomas" are reported to respond poorly to cytostatic drugs, but in most of the clinical studies a distinction between LMS and GIST has

not been made .[87,142,285,286,320] In case of metastatic disease of STS metastases are commonly present in the lungs. It has been found that lung metastases only have a better response to chemotherapy than metastases elsewhere .[321] Whether LMS and GIST differ in the development of liver metastases, which are associated with a poor prognosis, is at present unknown.

Multidrug resistance (MDR) i.e. resistance to numerous structurally unrelated natural product drugs such as the widely applied anthracyclines, has been associated with the overexpression of proteins like the MDR-1 gene product P-glycoprotein (P-gp) [99,108,288,297,322-326] and the multidrug resistance protein-1 (MRP_1) in drug resistant cell lines and neoplastic tissue .[107,108,287,289-291] Several studies indicate a possible role in clinical drug resistance for another protein: the major vault protein or lung resistance protein (LRP).[96,97,289,292-295] Expression of the MDR associated proteins P-gp, MRP_1 and LRP has not been studied in LMS and GIST, although this might reveal clinically valuable information.

This study, in a group of 29 LMS and 26 GIST patients, was performed to determine whether LMS and GIST are different clinical entities with regard to clinical behavior and MDR protein expression.

PATIENTS AND METHODS

Clinical data, i.e. patient characteristics, location of the tumor, presence of metastatic disease, follow-up status and survival data, were obtained by the analysis of the hospital medical records or information of the general practitioners. Between 1980 and 1998, 29 patients with a deep soft tissue LMS and 26 patients with a LMS of the digestive tract or GIST were evaluated in our hospital (Table 1a and b). Sixteen (55%) LMS were located in the lower extremity, 6 (21%) in the upper extremity, 4 (14%) in the retroperitoneum and 3 (10%) in other parts of the body. Twelve (46%) malignant GISTs were derived from the stomach, 6 (23%) from the small intestine, 6 (23%) from the large intestine, one (4%) from the esophagus and in one (4%) patient the tumor presented as a large intra-abdominal mass, suspected to derive from the stomach. Paraffin embedded tumor material was available of 26 patients with a LMS of soft tissue and 25 patients with a malignant GIST. Patients with superficial LMS i.e. originating from the cutis or subcutis were not included, since it is reported that they have a better prognosis than patients with deep soft tissue LMS .[8] For this study only previously untreated, primary tumors were used. In all cases the histological diagnoses were confirmed by central revision of haematoxylin-eosin stained paraffin sections with or without additional immuno-histological stains .[8,307] The 29 LMS were graded according to the grading system of Coindre et al. [29], which resulted in 8 grade I (28%), 14 grade II (48%) and 7 grade III (24%) LMS. A GIST was considered malignant if there was both mitotic activity and a tumor size of more than 5 cm in diameter, according to the recommendations of Suster .[307]

Table 1a. Patient characteristics, histology and MDR status of the 29 studied LMS patients

diagnosis	patient	age	gender	tumor grade	location	Follow -up	OS (months)	P-gp	MRP_1	LRP
LMS:										
	1	44	m	I	right thigh	NED	90	-	-	+
	2	44	m	I	left lower arm	NED	12	-	-	+
	3	44	f	I	right lower arm	DOD	26	+	-	+
	4	60	f	I	retroperitoneum	DOD	39	-	-	+
	5	64	m	I	left groin	NED	78	-	+	+
	6	73	f	I	left thigh	NED	45	-	+	+
	7	75	f	I	right ankle	NED	97	-	-	+
	8	83	f	I	retroperitoneum	DOD	9	-	-	+
	9	22	m	II	right lower leg	DOD	40	+	ND	+
	10	33	f	II	left lower leg	DOD	8	-	+	+
	11	34	m	II	Tongue	DOD	70	+	+	+
	12	54	f	II	left knee	NED	21	-	-	+
	13	54	f	II	Retroperitoneum	AWD	26	-	-	+
	14	55	m	II	Retroperitoneum	AWD	7	+	+	+
	15	59	m	II	right, supraclavicular	AWD	23	+	+	+
	16	62	f	II	right lower leg	DOD	51	-	+	+
	17	65	m	II	left lower leg	DOD	40	ND	-	-
	18	69	f	II	right buttock	NED	75	-	+	+
	19	71	m	II	right knee	DOD	10	+	-	+
	20	72	f	II	right upper leg	NED	12	-	-	+
	21	78	f	II	right buttock	DOD	136	ND	ND	ND
	22	80	f	II	left arm	DOD	19	-	ND	+
	23	20	f	III	left thigh	NED	121	ND	ND	ND
	24	40	f	III	left groin	DOD	22	+	-	+
	25	43	m	III	right upper leg	DOC	151	-	-	-
	26	45	m	III	right arm	DOD	4	-	-	-
	27	60	f	III	left lower leg	DOD	38	-	-	+
	28	70	m	III	right groin	DOD	39	ND	ND	ND
	29	81	f	III	right upper arm	DOD	14	+	-	+

LMS: soft tissue leiomyosarcoma , P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein; NED: no evidence of disease; AWD: alive with disease; DOD: died of disease; DOC: died of other causes; LOF: lost to follow-up; ND: not done

MDR expression

For detection of P-gp, the monoclonal antibody C494 (Signet Laboratories, Dedham MA) in a concentration of 120 µg/ml in phosphate-buffered saline (PBS) (Merck, Darmstadt, Germany) plus 1% bovine serum albumin (BSA) (Serva Electrophoresis GmbH, Hamburg, Germany) was used. For detection of MRP_1 the monoclonal antibody MRPr1 (provided by Dr. R.J.Scheper, Dept. of Pathology, Free University Hospital, Amsterdam) in a concentration of 20 µg/ml in 1% BSA/PBS was utilized. For detection of LRP the monoclonal antibody LRP (Transduction Laboratories, Los Angeles CA) in a concentration of 250 µg/ml in 1% BSA/PBS was used. As controls for immunohistochemal detection served cytospins of the well

documented cell lines A2780 and GLC4 and their corresponding multi-drug resistant sublines i.e. overexpressing P-gp (A2780 AD), MRP_1 (GLC4/ADR) and LRP (GLC4/ADR) [298,299], as well as paraffin embedded formalin fixed liver (P-gp), lung (MRP_1) and colon (LRP) tissue.

Paraffin sections (3 μm) were placed on positively charged glass slides and were dried. Immunohistochemistry was performed according to a method modified from Shi et al. .[177,178] Briefly, after heating on a hotplate, the slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After each of the subsequent steps, three 5 minutes washes in PBS were carried out. After three times heating in an autoclave for 5 minutes at 115° C in a 20 mM blocking reagens (Boehringer Mannheim, Mannheim, Germany) with pH=6.0, endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in PBS during 30 minutes. The slides were incubated with the specific antibody in 1% BSA/PBS (pH=7.4) for 1 hour at room temperature in a humidified chamber. The primary antibody was detected with a rabbit antimouse (DAKO, Glostrup, Denmark) (C494 and LRP) or rabbit antirat (DAKO, Glostrup, Denmark) (MRPr1) peroxidase labeled secondary antibody diluted in 1:50 + 1% human serum (type AB) followed by incubation with goat antirabbit conjugated peroxidase diluted in 1:50 +1% human serum (type AB). 3.3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis) with imidazole (Merck, Darmstadt, Germany) in PBS was used as the chromagen according to the manufacturer's instructions. After counterstaining with Mayer's haematoxylin, the slides were dehydrated through graded alcohols and mounted with coverslips.

The expression of P-gp, MRP_1 and LRP was assessed independently by two observers (B.P, H.H.) without knowledge of the histological diagnosis or clinical data. The P-gp, MRP_1, LRP proteins were studied in adjacent slides of the most viable parts of the tumor. Amount of P-gp, MRP_1 and LRP expression was semiquantitatively assessed by estimating the percentage of positive stained tumor cells and assessing staining intensity using a scale of 0 -3 (0: no staining, 1: very weak staining, 2: intermediate staining, 3: strong staining). Strong staining intensity was comparable to the staining intensity of the simultaneously stained positive control tissue. Both staining parameters were also combined into a so called histopath-score which is defined as: the estimated percentage of stained cells (0-100%) x the staining intensity (0-3) .[300] According to the literature, tumor samples were considered negative for expression of each of the proteins if ≤ 5% of the cells were positive [96] P-gp expression in 1 LMS and MRP_1 expression in 2 LMS could not be interpreted.

Statistics

A Mann Whitney test was carried out to analyze the differences in MDR parameters between LMS and GIST. To avoid the problem of multi-comparison in the analyses of P-gp, MRP_1 and LRP in relation with response, a Bonferroni correction factor of 3 was introduced. A Fisher exact test was used to compare MDR status and the frequency of metastases in both groups. To quantitate the degree of correlation between parameters, the Spearman's correlation coefficient was

computed. Actuarial survival curves were constructed by the Kaplan-Meier method in order to compare OS for the different patient groups in relation to MDR protein expression and metastatic disease. OS has been defined as the time between diagnosis and the moment of disease related death. Survival curves in the different groups were compared by the log-rank test. A two-tailed p-value of < 0.05 was considered to be significant. Statistical software SPSS 8.0 for Windows (SPSS Inc., Chicago IL) was used for statistical analysis.

Table 1b. Patient characteristics, histology and MDR status of the 26 studied GIST patients

Diagnosis	patient	age	gender	location	Follow-up	OS (months)	P-gp	MRP_1	LRP
GIST:									
	30	18	f	Stomach	LOF		+	+	+
	31	35	m	Small bowel	DOD	14	+	-	+
	32	38	m	Small bowel	DOD	32	+	+	+
	33	39	f	Small bowel	DOD	17	-	-	-
	34	46	m	Colon	DOD	2	+	+	+
	35	47	m	Colon	DOD	20	+	-	+
	36	50	m	Esophagus	NED	14	-	+	+
	37	51	m	Colon	AWD	33	+	-	+
	38	51	m	Stomach	DOD	49	+	-	+
	39	53	m	Stomach	DOD	28	+	-	+
	40	55	f	Stomach	DOD	30	-	+	+
	41	59	f	Colon	DOD	6	+	+	+
	42	62	f	Stomach	NED	16	-	+	+
	43	62	f	Small bowel	NED	13	+	+	+
	44	63	m	Stomach	LOF		+	+	+
	45	64	m	Stomach	NED	17	+	+	+
	46	66	f	Stomach	DOD	15	+	+	+
	47	66	m	Colon	NED	79	-	-	-
	48	67	f	Stomach	DOD	10	+	+	+
	49	67	m	Stomach	DOD	26	+	+	+
	50	67	f	Colon	DOC	16	-	+	+
	51	70	m	Small bowel	DOD	28	ND	ND	ND
	52	73	f	Small bowel	NED	23	-	+	+
	53	74	f	Stomach	NED	41	+	+	+
	54	78	f	intra-abdominal	DOD	7	-	-	-
	55	80	f	Stomach	DOD	8	+	+	+

GIST: malignant gastrointestinal stromal tumor, P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein; NED: no evidence of disease; AWD: alive with disease; DOD: died of disease; DOC: died of other causes; LOF: lost to follow-up; ND: not done

RESULTS

Patient characteristics and clinical outcome:

As shown in Tables 1a and 1b, the LMS group consisted of 12 men and 17 women, with a mean age of 57 years (SD: 16.2, range: 20-83 years). The GIST group consisted of 13 men and 13 women, with a mean age of 58 years (SD: 14.7, range: 18-80 years). Gender and age were equally distributed between both groups.

Overall survival of the 29 patients with a LMS (mean: 72; median: 40 months) was significantly ($p<0.05$) better than the overall survival of the 26 patients with a GIST (mean: 31; median: 28 months) (Fig 1). The 2-yrs and 5-yrs OS in the group of LMS patients was 69.7% and 35.5%, whereas the 2-yrs and 5-yrs OS was significantly lower in the group of GIST patients i.e. 24.5% and 12.2%, respectively.

In the GIST group, OS was not significantly influenced by the location of the primary tumor i.e. patients with malignant GIST located in the stomach had a mean OS of 29 months (median: 28 months), whereas these values for small intestine and large intestine GIST were 24 (median: 28) and 41 (median: 20) months.

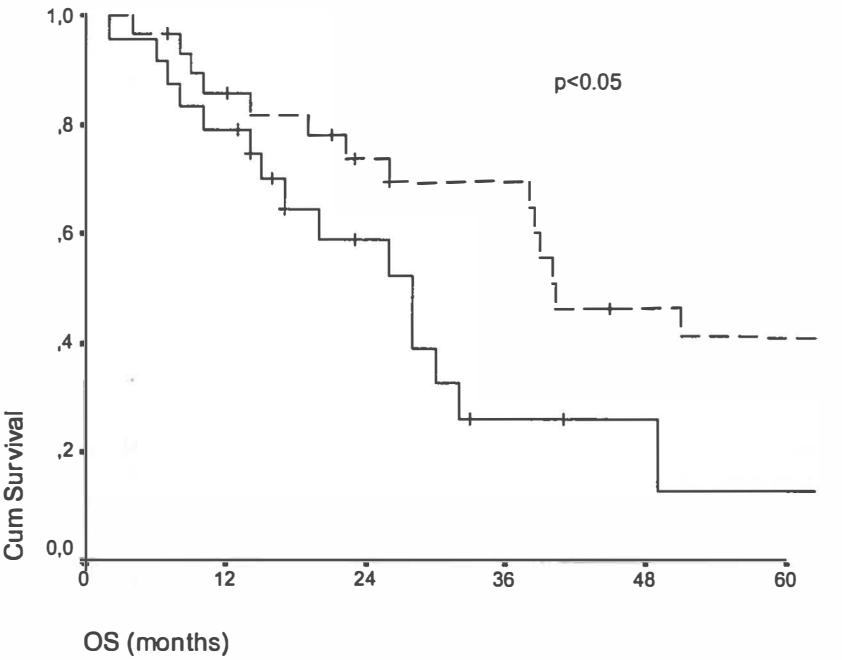


Figure 1.
Overall survival of the evaluable LMS and GIST patients

Due to the long period in which the patients were studied, it was not possible to determine whether there had been distant metastases in 3 LMS cases and 9 GIST cases. Of the remaining 26 LMS patients, 15 developed metastases: lung metastases were found in 13 patients and in skin metastases in two patients (Table 2). None of the LMS patients developed liver metastases as the first metastatic site, but four patients developed liver metastases subsequent to other distant metastases. Of the remaining 17 GIST patients, 9 patients developed distant metastases: liver metastases were found in 5 patients, intra-abdominal metastases in 2 patients and regional lymph node metastases in one patient. One GIST patient developed intrathoracal metastases, presumably per continuitatem with intra-abdominal metastasis. Although the frequency of metastases in the evaluable LMS (15/26) and GIST (9/17) patients was comparable, the liver as the first metastatic site was more involved in GIST patients ($p<0.01$), while the lungs as the first metastatic site were more involved in patients with a deep seated LMS ($p<0.001$).

Table 2. Distant metastases in LMS and GIST.

	LMS n (%)	GIST n (%)
no metastases	11 (38%)	8 (30%)
distant metastases	15 (52%)	9 (35%)
unknown	3 (10%)	9 (35%)
lung metastases	13 (50%)[a]	1[b] (11%)[a]
- lung metastases only	8 (31%)	1[b] (11%)
- lung as primary metastatic site	13 (50%)	1[b] (11%)
liver metastases	4 (15%)	5 (56%)
- liver metastases only	0	2 (22%)
- liver as primary metastatic site	0	5 (56%)
intra-abdominal metastases	0	4[c] (44%)
skin metastases	5[d] (19%)	0
elsewhere	0	1 (11%)

LMS: soft tissue leiomyosarcoma , GIST: malignant gastrointestinal stromal tumor

[a] percentage of patients who were evaluable for the analysis of metastatic disease

[b] this patient developed intrathoracal metastases, presumably per continuitatem with intra-abdominal metastasis

[c] 2/4 patients had intra-abdominal metastases as primary metastatic site

[d] 2/5 patients had skin metastases as primary metastatic site

P-gp, MRP_1 and LRP expression in LMS and GIST

P-gp and MRP_1 were significantly more expressed in the GIST than in the LMS: P-gp positive LMS were found in 8/26 (31%) cases and P-gp positive GIST could be identified in 17/25 (68%) cases ($p<0.05$). MRP_1 positive LMS were found in 8/26 (31%) cases and MRP_1 positive GIST could be identified in 17/25 (68%) cases ($p<0.05$)(Table 3). As shown in Fig 2, the amount of P-gp and MRP_1 expressing tumor cells was significantly higher in GIST than in LMS (both: $p<0.05$). When the percentage positive cells were combined with staining intensity into a histopath score, the differences in MRP_1 expression were even more pronounced (Table 4). LRP expression did not differ between LMS and GIST, but was more often expressed than P-gp and MRP_1 (both: $p<0.001$). A minority of LMS (12%) and GIST (12%) had no MDR expression, while 12% of the LMS and 48% of the GIST expressed all three MDR-proteins ($p<0.01$). Numbers of tumors with co-expression of P-gp, MRP_1 or LRP are shown in Table 3.

Table 3. MDR protein status of the analyzed 26 LMS and 25 GIST.

	LMS	GIST
P-gp +	8 (31%)	17 (68%)
MRP +	8 (31%)	17 (68%)
LRP +	23 (88%)	22 (88%)
P-gp + and MRP +	3 (12%)	12 (48%)
P-gp + and LRP +	8 (31%)	17 (68%)
MRP + and LRP +	8 (31%)	17 (68%)
no MDR expression	3 (12%)	3 (12%)
expression of one MDR protein	10 (38%)	0 (0%)
co-expression of two MDR proteins	10 (38%)	10 (40%)
co-expression of all three MDR proteins	3 (12%)	12 (48%)

LMS: soft tissue leiomyosarcoma , GIST: malignant gastrointestinal stromal tumor,
P-gp: P-glycoprotein, MRP_1: multidrug resistance protein,
LRP: lung resistance protein

A correlation (r: 0.33; $p<0.05$) was found between P-gp and MRP_1 expression (both percentage positive cells and histopath score) in the whole group of tumors, but not in the subgroups of LMS and GIST. LRP expression was not related to P-gp or MRP_1 expression. P-gp, MRP_1 and LRP expression was neither correlated with tumor grade nor with mitotic activity.

OS in relation with MDR status in LMS and GIST

When LMS patients were compared with GIST patients regarding their OS and MDR status, it was found that patients with P-gp positive LMS tended to have a shorter OS than patients with P-gp negative LMS (Table 5). P-gp expression did not influence survival in GIST. No differences in survival in either LMS patients or GIST patients were observed regarding MRP_1 or LRP status. Co-expression did not influence OS.

Table 4. Summary of P-gp, MRP_1 and LRP expression in the 26 LMS and 25 GIST.

MDR protein	histology	percentage positive cells					histopath score*				
		mean	SD	median	range	p	mean	SD	median	range	p
P-gp	LMS	13.4%	23.0%	5%	0-80%	0.01	22.5	38.5	10	0-160	0.03
	GIST	38.4%	33.9%	30%	0-90%		81.4	92.1	30	0-270	
MRP_1	LMS	13.3%	22.9%	0%	0-80%	0.04	17.7	33.0	0	0-140	0.02
	GIST	35.4%	32.1%	20%	0-90%		64.6	62.9	40	0-180	
LRP	LMS	58.5%	27.3%	60%	0-90%	0.59	111.2	73.6	90	0-270	0.68
	GIST	53.6%	30.7%	60%	0-90%		102.8	59.2	120	0-180	

*histopath score: percentage positive cells x staining intensity (0-3), LMS: soft tissue leiomyosarcoma , GIST: malignant gastrointestinal stromal tumor,
P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein;
p: p - values (adjusted for multiple comparison)

Table 5. MDR status and OS in both LMS and GIST.

	LMS	GIST
	OS (months) median (mean)	OS (months) median (mean)
all cases	40 (72)*	28 (31)*
P-gp +	26 (33)	26 (25)
P-gp -	51 (88)	30 (40)
MRP_1 +	70 (61)	26 (24)
MRP_1 -	38 (61)	20 (35)
LRP +	40 (52)	28 (27)
LRP -	40 (65)	17 (34)

LMS: soft tissue leiomyosarcoma , GIST: malignant gastro-intestinal stromal tumor, P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein , *$p<0.05$

DISCUSSION

Immunohistochemical and molecular (cyto)genetic findings have brought evidence that GIST have to be considered as a specific group of neoplasms .[307,327] GIST have been diagnosed as smooth muscle tumors of the digestive tract for many years and the studies evaluating clinical outcome and treatment response have classified them as "leiomyosarcomas of the digestive tract". A number of studies have reported the chemoresistance of the so-called leiomyosarcomas and the poor prognosis of patients with an abdominal "leiomyosarcoma", but it is not clear whether these non-responding malignant smooth muscle neoplasm are GIST, LMS or uterine LMS .[87,142,285,286,320] The results of a recent study by Edmonson et al. [328], in which malignant GIST and other "leiomyosarcomas" were treated with dacarbazine, mitomycine, doxorubicin and cisplatin, indicate that GIST are more chemotherapy resistant than other neoplasms with smooth muscle differentiation.

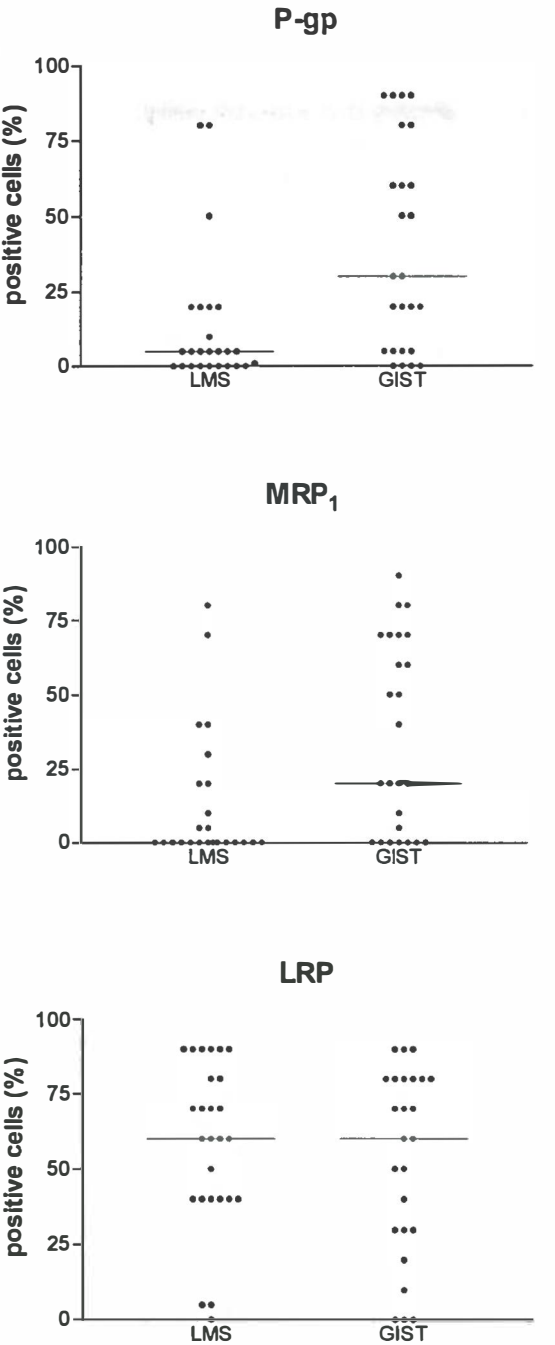


Figure 2.
P-gp, MRP_1 and LRP expression in both LMS and malignant GISTs.

The present study showed that equal percentages of LMS and GIST develop distant metastases. However, the frequency of lung metastases in metastasized LMS was 87% and liver metastases 27%, whereas in 11% of the metastasized GIST patients lung metastases developed and in 56% liver metastases were found. This strengthens the idea that most studies reporting the high incidence of hepatic metastases in "leiomyosarcomas" [87,329] are in fact evaluating malignant GIST and that (metastatic) LMS might be more drug sensitive than has been suggested in earlier studies.

No previous studies were performed in which GIST were compared with LMS of deep soft tissue regarding clinical outcome and the presence of drug resistance associated proteins. The results of the current study not only indicate that patients with a LMS have a better prognosis than patients with malignant GIST, but revealed clinically important evidence that malignant GIST express P-gp and MRP_1 more frequently than LMS.

The OS was evaluated without information of the adequacy of the primary surgical treatment and of additional treatment, since in the accrual of a relatively large amount of patient and tumor data of relatively rare neoplasms, a 19 years period is involved, making the importance of the additional information of the various applied treatment regimens questionable. Although LMS patients have a worse prognosis than other STS patients [8], OS in LMS patients is significantly better than in GIST patients. It can be concluded that although LMS are aggressive STS, they still have a better clinical behavior than malignant GIST. Several studies reported the 5 yrs survival rate in GIST, ranging from 20% to 78% [318], but did not clearly indicate whether these GIST were malignant or benign. In our study we combined the presence of mitotic activity per 2 mm^2 and a tumor size of more than 5 cm in diameter, to select the malignant GIST .[307] Several studies in large series of GIST patients did report a better survival of patients with GIST located in the stomach or esophagus than GIST derived from the small or large intestine, but this could not be confirmed in our study with a relatively small number of patients .[308,318] It should be emphasized that only deep seated LMS were included in this study and that (sub)cutaneaous LMS were not examined, since these superficial tumors have a better prognosis than the LMS of the deep soft tissues, probably because they are small when detected and adequately excised .[8,330,331]

The finding that GIST have significantly more expression of P-gp and MRP_1 than LMS, is not only another indication that GIST is a different tumor, but is also of therapeutic importance. In most STS, doxorubicin based regimens are administered in metastatic disease, but also in the adjuvant setting .[281,282,332-338] Edmonson et al [328] showed that GIST are more chemotherapy resistant than other malignant smooth muscle tumors when doxorubicin based chemotherapy was used. However, doxorubicin resistance is related to P-gp and MRP_1 [289]and the results of our study strongly imply that especially in malignant GIST with high expression of both P-gp and MRP_1, doxorubicin is not indicated and new non-MDR related cytostatic agents are awaited. Both P-gp and MRP_1 expression is frequently detected in normal tissue of the digestive tract [339,340], which could explain their expression in GIST. Since P-gp

expression and MRP_1 expression was neither correlated to tumor grade in LMS nor to the mitotic activity in both LMS and GIST, it appears that both MDR parameters are not related to tumor aggressiveness and are more related to the histological type and the tissue they derive from as has been demonstrated in other studies .[340-346]

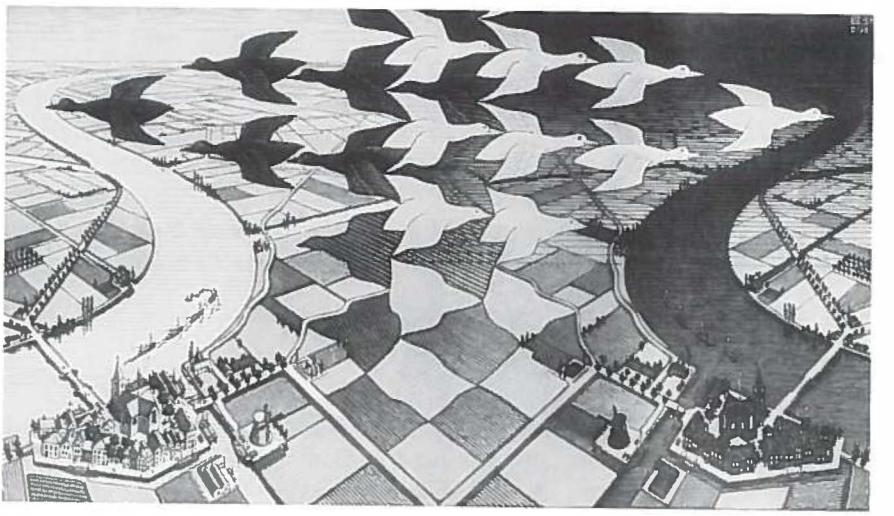
High LRP expression was found in both LMS and malignant GIST and LRP expression did not have prognostic value in both groups. The high expression of LRP might be due to the fact that LRP is present in the epithelial tissues of digestive tract. Although the distribution of LRP expression in normal tissues has not been studied extensively, it was found to be present in muscle tissue , but not in muscle tissue with high MRP_1 expression or in normal smooth muscle .[292,347] In the present study, no inverse correlation between MRP_1 and LRP expression was found, indicating that LRP expression has not been decreased by simultaneous MRP_1 expression in these smooth muscle neoplasms. On microscopic examination the LRP staining was most cytoplasmic and normal smooth muscle showed no clear staining. The exact function of LRP is not yet understood and, although there is evidence that LRP acts as an intracytoplasmic transporter of cytotoxic agents. In contrast to acute myeloid leukemia and ovarian cancer where LRP expression has been found an independent prognostic factor for response to chemotherapy and outcome [96,97,295], not much is known about LRP expression in STS and its prognostic role in STS is still unclear.

Other studies have shown the clinical importance of P-gp and MRP_1 (co-) expression in sarcomas in general .[98-103,107,108] In this study we not only determined the percentage of stained cells, but also incorporated the staining intensity in our evaluation of the tumors into a histopath score. In this way tumors with high staining intensity could be distinguished from moderate stained tumors even if the percentage of tumor cells was the same. This way of evaluating immunohistological stained sections reveals a broader range of staining results than the estimation of MDR positive cells alone. It was found that both the estimation of the amount of positive cells as well as the histopath score was highly comparable. The cutoff level of 5% was used to assess whether tumors were P-gp, MRP_1 or LRP positive or negative. A cutoff level has been introduced earlier [96] since immunohistological assessment cannot determine accurately if less than 5% stained cells in a minority of tumor specimens are indeed malignant tumor cells or pre-existent normal tissue, blood cells or endothelial cells.

In conclusion, LMS patients have a better survival than GIST patients and LMS have a less pronounced drug resistance pattern than GIST. Therefore, studies evaluating chemotherapeutic regimens should differentiate between LMS and malignant GIST. The observed high expression of P-gp and MRP_1 in GIST might explain their poor response to standard (anthracycline-based) chemotherapy. Although an equal amount of the LMS and the GIST develop distant metastases, LMS predominantly metastasize to the lungs, while GIST tend to spread to the liver and the abdominal cavity. Further studies are warranted to determine whether new cytostatic agents are effective in GIST and might be used in case of metastatic disease.

Chapter 9

P-gp, MRP_1 AND LRP EXPRESSION RELATED TO TREATMENT RESPONSE AND CLINICAL OUTCOME IN ADULT SOFT TISSUE SARCOMA PATIENTS TREATED WITH EPIRUBICIN, VINDESINE AND IFOSFAMIDE (EVI)



B.E.C. Plaat, W.M. Molenaar, H. Hollema, M.F. Mastik, H.H. Luu, H.J. Hoekstra, E.G.E. de Vries, E. van den Berg, N.H. Mulder, W.T.A. van der Graaf

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SUMMARY

Most adult patients with disseminated (soft tissue) sarcomas (STS) still have a poor clinical outcome. Multidrug resistance (MDR) has been associated with the expression of P-glycoprotein (P-gp), multidrug resistance protein-1 (MRP_1) and lung resistance protein (LRP), but has not been studied intensively in a uniformly treated group of adult STS patients. In 28 adult STS patients with metastatic disease or irresectable tumor, treated with epirubicin, vindesine and ifosfamide (EVI) we evaluated the expression of P-gp, MRP_1 and LRP, to determine the relation with treatment response and clinical outcome. MDR-protein expression was semiquantitatively assessed after immunohistochemical detection in paraffin embedded tumor material obtained before start of chemotherapy.

P-gp positivity was observed in 63%, MRP_1 positivity in 57% and LRP positivity in 73% of the tumors. In 93% of the tumors at least one analyzed MDR-protein was present. Co-expression was found in 75% of the tumors and 20% were positive for all three MDR proteins. Tumor response was observed in 16 patients (3 CR, 13 PR) and no response in 11 patients (8 SD, 3 PD). MDR-protein expression was not associated with treatment response. Overall survival (OS) (median: 14 months) and progression free survival (PFS) (median: 8 months) were not associated with the expression of P-gp, MRP_1 or LRP. However, patients with sarcomas co-expressing two or three drug resistance proteins (n = 21) tended to have a worse median PFS (7 months) than those without co-expression (11 months) (p=0.08). In conclusion, the majority of adult STS expresses P-gp, MRP_1 and/or LRP. In the individual patient with a metastasized STS, the assessment of P-gp, MRP_1 or LRP expression cannot predict response to EVI treatment. There is a tendency that co-expression of P-gp, MRP_1 and/or LRP is related with a worse PFS.

INTRODUCTION

Sarcomas are a heterogeneous group of malignant tumors which arise from tissue of mesenchymal origin and account for approximately 2 % of all malignancies. They originate in the soft tissues i.e. soft tissue sarcomas (STS) or bone (e.g. Ewing's sarcomas and osteosarcomas). STS tend to spread hematogeneously, predominantly to the lungs, in up to 30% of the patients [8,142,348]. Chemotherapy is the treatment of choice in adult STS patients with diffuse distant metastases or advanced inoperable tumors [86]. Doxorubicin is the most extensively applied cytotoxic drug in STS, alone or in combination with other drugs, and can be considered as the standard chemotherapeutic agent in STS resulting in response rates of up to 35% [281,282,332,335]. The addition of ifosfamide, with single agent response rates of 26%, and etoposide has shown to increase response rates to 46% [86,87,283,284,335,349]. The efficacy of vinca-alkaloids has been examined in polychemotherapy schedules in STS [282]. Overall, the response rates in adult sarcoma patients to various chemotherapeutic regimens are around 26% with a median survival time of 51 weeks [87].

Multidrug resistance (MDR) is cross resistance to a number of functionally and structurally distinct drugs such as the anthracyclines (doxorubicin, epirubicin), the vinca-alkaloids (vincristine, vindesine) and epipodophyllotoxins (etoposide). MDR can be caused by one or more mechanisms i.e. increased expression of energy dependent efflux pumps such as P-glypoprotein (P-gp) and the multidrug resistance protein-1 (MRP_1), or increased expression of the lung resistance related protein (LRP) [287-290,292-294,350,351]. P-gp has been found localized on the cell membrane, MRP_1 has been localized both cytoplasmic and on the cell membrane [103,305]. The spectrum of resistance for cytostatic agents relevant in sarcomas does not differ much between P-gp and MRP_1 . LRP, which was identified as the human major vault protein is located intracytoplasmic and has been considered to play a role in the transport between nucleus and cytoplasm [289,292-294]. Until now, the role of LRP in MDR is less clear, although some studies in ovarian cancer, multiple myeloma and leukemia indicated a possible role in clinical drug resistance [96,97,295,296].

In sarcomas, P-gp expression was found to be associated with poor response to chemotherapy in childhood rhabdomyosarcomas [102,352], although the predictive value has been weakened later [103], and Ewing's sarcomas [98] as well as with poor prognosis in osteosarcomas [99] and adult STS [100,101]. MRP_1 has been detected in Ewing's sarcoma [107] and co-expression of MRP_1 and P-gp was associated with tumor grade in STS [108]. MRP_1 has not been evaluated in relation to chemotherapeutic response or clinical outcome in STS patients. LRP expression has not been studied intensively in sarcomas. Until now, the combined expression of P-gp, MRP_1 and LRP has not been studied with respect to clinical outcome in a uniformly chemotherapeutically treated group of adult patients with metastatic STS.

This study was performed to evaluate the expression of P-gp, MRP_1 and LRP in adult STS patients and to determine a possible relation with response and survival following polychemotherapy (EVI).

PATIENTS AND METHODS

Patients

Between January 1992 and January 1998, 31 adult patients were treated with standard chemotherapy consisting of EVI. Patients were eligible for treatment with EVI if they had a histologically proven malignant mesenchymal tumor of the soft tissues with well documented distant metastases or an irresectable tumor not amenable to isolated limb perfusion. Patients had to be between 16 years and 75 years of age, had to have a World Health Organization (WHO) performance status of 0, 1 or 2 and should not have received prior chemotherapy.

Since in three patients paraffin embedded tumor material was not available, the studied group consisted of 28 patients (mean age 44.1, median: 44, SD: 14.6 , range: 16-67 years). Twenty seven patients were treated for metastatic STS. One patient (case 27) received EVI for an irresectable primary STS and this patient was not included in the survival analyses. Since in one patient the treatment response

could not be assessed, 27 patients were evaluable for analysis of treatment response. For this study, tumor material of the last tumor presentation obtained before the start of chemotherapy was used: 19 primary tumors and 9 distant metastases. The period between the last obtained and available pretreatment sample and the start of the EVI treatment ranged from 0 - 52 months (median: 4.1, mean: 9.6, SD: 12.9 months). Patient characteristics, histology and clinical data are presented in Table 1.

Table 1. Patient characteristics, histology and clinical outcome

Pat	sex	age	diagnosis	tumor grade	treatment response	OS[a] (months)	PFS[b] (months)
1	F	39	leiomyosarcoma	III	PD[e]	13	0
2	M	55	synovial sarcoma	II	SD	13	8
3	M	53	malignant peripheral nerve sheath tumor	II	NE	20	20
4	F	58	alveolar soft part sarcoma	II	PD	1	0
5	F	59	leiomyosarcoma	II	SD	38	11
6	M	22	synovial sarcoma	III	PR	15	12
7	F	61	leiomyosarcoma	III	PR	14	11
8	M	53	angiosarcoma	II	PD	7	0
9	F	60	leiomyosarcoma	I	PR	39	35
10	M	62	malignant peripheral nerve sheath tumor	II	SD	11	8
11	F	42	MGCT[c]	III	PR	10	6
12	F	39	rhabdomyosarcoma	II	PR	11	7
13	F	19	rhabdomyosarcoma	I	CR	18	16
14	M	23	sarcoma not otherwise specified	II	SD	6	4
15	F	52	myxoid liposarcoma	I	PR	34	21
16	F	41	sarcoma not otherwise specified	III	PR	6	5
17	F	40	uterine leiomyosarcoma	-	SD	20	7
18	F	24	extraskeletal Ewing's sarcoma	III	PR	13	7
19	M	67	malignant fibrous histiocytoma	II	CR	20	10
20	F	16	rhabdomyosarcoma	II	CR	22	11
21	F	52	epithelioid sarcoma	III	SD	7	4
22	F	27	sarcoma not otherwise specified	III	PR	6	5
23	M	59	leiomyosarcoma	II	SD	16+	10+
24	M	45	rhabdomyosarcoma	III	PR	9	6
25	F	32	synovial sarcoma	II	PR	12+	12+
26	F	43	myxoid liposarcoma	II	PR	13+	13+
27	F	41	sarcoma not otherwise specified	II	PR	16+	16+
28	M	51	malignant GIST[d]	-	SD	12+	12+

[a] OS: overall survival, [b] PFS: progression free survival,
[c] MGCT: malignant giant cell tumor of tendons and aponeuroses, [d] GIST: gastrointestinal stromal tumor,
[e] CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease,
NE: not evaluable, + alive with disease or (patient 27) no evidence of disease

Histology

In all cases the histological diagnosis was made on haematoxylin-eosin stained paraffin sections with or without additional immunohistological stains. The STS were classified according to Enzinger and Weiss [8] and 26 could be graded according to the grading system of Coindre et al. [29]. This resulted in 3 grade I (11%), 14 grade II (54%) and 9 grade III (35%) STS (Table 1).

MDR expression

For detection of P-gp, the monoclonal antibody C494 (Signet Laboratories, Dedham MA) in a concentration of 120 µg/ml in phosphate-buffered saline (PBS) (Merck, Darmstadt, Germany) plus 1% bovine serum albumin (BSA) (Serva Electrophoresis GmbH, Hamburg, Germany) was used. For detection of MRP_1 the monoclonal antibody MRPr1 (kindly provided by Dr R.J.Scheper, Free University Hospital, Amsterdam) in a concentration of 20 µg/ml in 1% BSA/PBS was utilized. For detection of LRP the monoclonal antibody LRP (Transduction Laboratories, Los Angeles CA) in a concentration of 250 µg/ml in 1% BSA/PBS was used. As controls for immunohistochemal detection served cytospins of the well documented cell lines A2780 and GLC4 and their corresponding multi-drug resistant sublines i.e. overexpressing P-gp (2780 AD), MRP_1 (GLC4/ADR) and LRP (GLC4/ADR) [298,299], as well as paraffin embedded formalin fixed liver (P-gp), lung (MRP_1) and colon (LRP) tissue.

Paraffin sections (3 µm) were placed on positively charged glass slides and were dried. Immunohistochemistry was performed according to a method modified from Shi et al. [177,178]. Briefly, after heating on a hotplate, the slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After each of the subsequent steps, three 5 min washes in PBS were carried out. After three times heating in an autoclave for 5 min at 115° C in a 20 mM blocking reagens (Boehringer Mannheim, Mannheim, Germany) with pH=6.0, endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in PBS during 30 min. The slides were incubated with the specific antibody in 1% BSA/PBS (pH=7.4) for 1 h at room temperature in a humidified chamber. The primary antibody was detected with a rabbit antimouse (DAKO, Glostrup, Denmark) (C494 and LRP) or rabbit antirat (DAKO, Glostrup, Denmark) (MRPr1) peroxidase labeled secondary antibody diluted in 1:50 + 1% human serum (type AB) followed by incubation with goat antirabbit conjugated peroxidase diluted in 1:50 +1% human serum (type AB). 3.3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis) with imidazole (Merck, Darmstadt, Germany) in PBS was used as the chromagen according to the manufacturer's instructions. After counterstaining with Mayer's haematoxylin, the slides were dehydrated through graded alcohols and mounted with coverslips.

The expression of P-gp, MRP_1 and LRP was assessed independently by two observers (B.P, H.H.) without knowledge of the EVI treatment response or patient survival. The P-gp, MRP_1, LRP proteins were studied in adjacent slides of the most viable parts of the tumor. Amount of P-gp, MRP_1 and LRP expression was

semiquantitatively assessed by estimating the percentage of positive stained tumor cells and assessing staining intensity using a scale of 0 -3 (0: no staining, 1: very weak staining, 2: intermediate staining, 3: strong staining). Strong staining intensity was comparable to the staining intensity of the simultaneously stained positive control tissue. Both staining parameters were also combined into a so called histopath-score which is defined as: the estimated percentage of stained cells (0-100%) x the staining intensity (0-3) [300]. According to the literature, tumor samples were considered negative for expression of each of the proteins if ≤ 5% of the cells in the tumor section were positive [96]. Since not enough paraffin embedded tumor material was available, it was not possible to determine P-gp expression in one case and LRP expression in two cases.

Chemotherapy treatment

The treatment protocol consisted of a bolus of 3 mg/m^2 vindesine and 120 mg/m^2 epirubicin given intravenously (iv) on day 1, followed by a 1 h infusion of 1.2 g/m^2/day ifosfamide, combined with mesna, on days 2 to 6. Courses were repeated every three weeks. Patients showing a response or stable disease received two additional courses until a maximum of 6 courses. Therapy was ended when disease progression was documented, unacceptable toxicity occurred or patients refused further treatment.

Assessment of tumor response

Tumor response was assessed after every two courses. Complete response (CR) was defined as the disappearance of all signs of disease for at least 4 weeks. A reduction by at least 50% in the sum of the products of the largest perpendicular diameters of all measurable lesions was defined as a partial response (PR). Stable disease (SD) was defined as a situation with less than 50% decrease or less than 25% increase and progressive disease (PD) as more than 25% increase in the sum of the aforementioned diameters. Response was scored according to WHO criteria [353] depending on their tumor localization either by physical examination or radiographically. The product of the largest perpendicular diameters of the tumor was used as response parameter. Overall survival (OS) was defined as the period between the start of the chemotherapy and the patient's death or the last follow-up visit. Progression free survival (PFS) was defined as the period between the start of the treatment and disease progression.

Statistics

A Mann Whitney test was carried out to analyze the differences in MDR parameters between poor and good responders. To avoid the problem of multi-comparison in the analyses of P-gp, MRP_1 and LRP in relation with response, a Bonferroni correction factor of 3 was introduced. To quantitate the degree of correlation between parameters, the Spearman's rank test was used. Actuarial survival curves were constructed by the Kaplan-Meier method in order to compare OS and PFS for the different patient groups in relation to MDR protein expression

and response. Survival curves in the different groups were compared by the log-rank test. A two-tailed p-value of < 0.05 was considered to be significant. Statistical software SPSS 8.0 for Windows (SPSS Inc., Chicago IL) was used for statistical analysis.

RESULTS

P-gp expression, i.e. >5% of the cells in the tumor section were positive, was found in 17/27 (63%) of the EVI treated sarcomas. Median percentage P-gp positive cells was 60% and median P-gp histopath score was 60 (Table 2). In 12 of these tumors a low diffuse cytoplasmic and membranous staining was observed, while in 2 cases a moderate and in 3 tumors a strong staining was observed. MRP_1 positive sarcomas were found in 16/28 (57%) cases. The median percentage of MRP_1 expressing cells was 10% and the median MRP_1 histopath score was 15. In 9 of these tumors a low diffuse cytoplasmic staining was observed, while in 2 cases a moderate and in 3 tumors a strong cytoplasmic and membranous staining was observed. LRP positive sarcomas were found in 19/26 (73%) of the patients. Median percentage LRP positive cells was 30%. Median LRP histopath score was 35. In 10 of these tumors a low diffuse cytoplasmic was observed, while in 8 cases a moderate and in 1 tumor a strong staining was observed.

Table 2. Summary of P-gp, MRP_1 and LRP expression in the EVI treated tumors

	n	median	mean	SD	range
positive cells:					
P-gp[b]	27	60%	47%	41.9%	0%-100%
MRP_1[c]	28	10%	31%	33.9%	0%-90%
LRP[d]	26	30%	35%	32.5%	0%-90%
histopath score[a]:					
P-gp[b]	27	60	74	89.5	0-300
MRP_1[c]	28	15	55	79.2	0-270
LRP[d]	26	35	59	71.2	0-270

[a]histopath score: % positive cells x staining intensity (0-3), [b]P-gp: P-glycoprotein, [c]MRP_1: multidrug resistance protein, [d]LRP: lung resistance protein

Twenty-six of the 28 tumors (93%) did express one or more of the MDR proteins. Twenty-one of the 28 tumors (75%) were co-expressing two or three MDR proteins. Co-expression of P-gp and MRP_1 was found in 10/27 cases (37%), P-gp and LRP 11/25 (44%) cases, MRP_1 and LRP in 12/26 (46%) cases and expression of all three MDR proteins was observed in 5/25 (20%) of the sarcomas. Two sarcomas (7%) were negative for all three MDR proteins. The expression of P-gp, as assessed by both the percentage of positive cells and the histopath score, did not correlate with to the expression (positive cells and histopath score) of either MRP_1 or LRP. Expression of MRP_1 was not correlated to LRP expression as assessed by both the percentage of positive cells and the histopath score.

A tumor response was observed in 59% (3 CR, 13 PR) of the 27 patients evaluable for response. No response was observed in 41% (11/27) of the patients (3 PD, 8 SD). As shown in Table 3, P-gp, MRP_1 or LRP expression did not differ between the patients with or without a response on EVI treatment. Although not statistically significant, median MRP_1 and LRP expression was lower in the responders. No correlation was found between response and P-gp, MRP_1 or LRP expression as assessed by percentage of positive cells or histopath score (Figure 1).

Table 3. P-gp, MRP_1 and LRP expression in sarcomas of patients with a response (n: 16) and no tumor response (n: 11) on EVI.

	percentage positive cells			histopath score[a]		
	median	mean	SD	median	mean	SD
P-gp[b]						
response	80%	60%	38.9%	90	97	93.1
no response	5%	33%	41.7%	10	50	81.9
MRP_1[c]						
response	0%	26%	34.8%	0	43	74.7
no response	40%	40%	33.2%	40	75	87.6
LRP[d]						
response	20%	32%	33.7%	20	51	74.8
no response	35%	41%	31.3%	50	74	66.1

[a]histopath score: percentage positive cells x staining intensity (0-3), [b]P-gp: P-glycoprotein, [c]MRP_1: multidrug resistance protein, [d]LRP: lung resistance protein

Median OS in the EVI treated STS was 14.0 months, whereas median PFS was 8.0 months. OS was 14.7 months for responders and 12.7 months for non-responders (N.S.) (Figure 2). Median PFS was better for responders (11.0 months) than for non-responders (7.0 months) (p=0.02). Patients with P-gp, MRP_1 or LRP negative STS did not have a better median OS as compared to patients with P-gp, MRP_1 or LRP positive tumors: 13 (P-gp-), 15 (P-gp+), 11 (MRP_1 -) and 18 (MRP_1 +), 13 (LRP-) and 14 (LRP+) months. Also median PFS was not associated with MDR: 8 (P-gp-) and 7 (P-gp+), 8 (MRP_1 -) and 8 (MRP_1 +), 7 (LRP-) and 10 (LRP+) months. However, expression of two or more MDR proteins (i.e. co-expression) did negatively influence OS, but differences in OS were not statistically significant. PFS was notably shorter for the patients with tumors co-expressing two or three MDR proteins (p=0.08) (Figure 3).

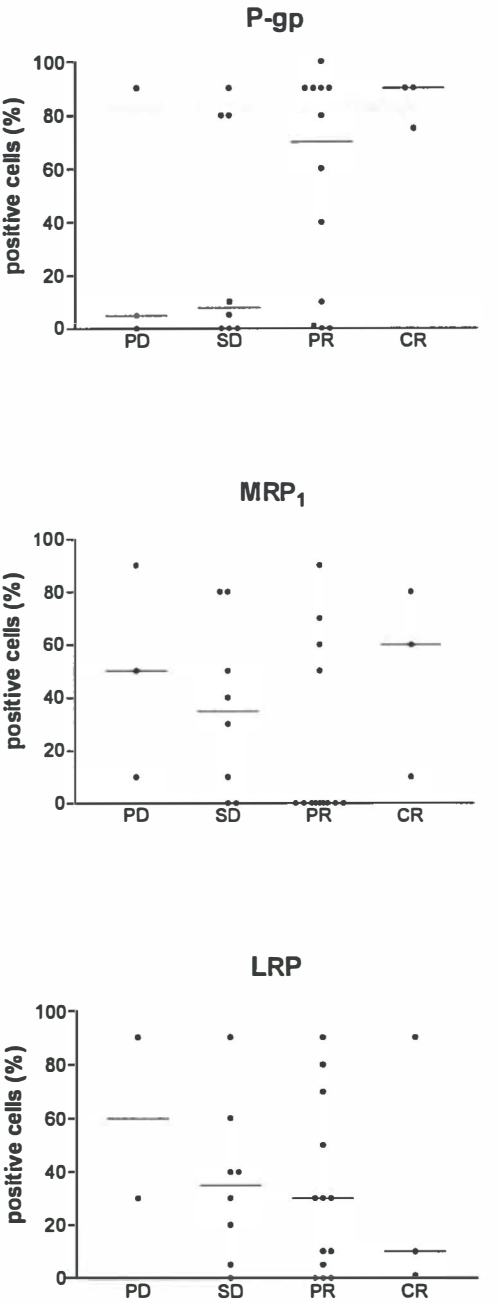


Figure 1
Tumor reponse (PD: progressive disease, SD: stable disease, PR: partial response, CR: complete response) related to (a) P-gp, (b) MRP_1 and (c) LRP expression.

DISCUSSION

Although doxorubicin or epirubicin and ifosfamide have shown activity in the treatment of STS, it should be envisaged that still the majority of STS patients do not respond to chemotherapy [87]. This urges not only the need for more active cytostatic agents but also for defining tumor biological characteristics with prognostic significance. This should lead to more patient or tumor-tailored therapy which might be in favor of enhancing response to treatment but also of withholding useless therapy with known side-effects. Therefore it was decided to translate the expression of P-gp, MRP_1 and LRP in chemotherapeutic naive adult STS, obtained from the last presentation before the start of EVI treatment, into a clinical setting, i.e. response to treatment, OS and PFS.

In this study it was shown that most of the chemotherapy naive sarcomas express P-gp, MRP_1 or LRP. Furthermore, co-expression of two or three MDR proteins is present in 75% of the STS and the expression of P-gp, MRP_1 and LRP did not influence response to EVI. It has to be considered that epirubicin and vindesine are typical P-gp related cytostatic agents, whereas ifosfamide is not. So the contribution of the individual compounds in this polychemotherapy schedule is difficult to establish. In any way, no single parameter seems to overrule the others.

P-gp expression was common (63%) in STS and was found in both the cytoplasm and cell membrane, as described earlier [103]. Levine et al. [101] found 24/50 (48%) P-gp positive STS using RT-PCR for MDR-1 mRNA and 51% P-gp positive STS using immunohistochemistry with the UIC-2 antibody. However, in the latter study 7 cases were intermediate malignant desmoid tumors, only 42% of the patients received chemotherapeutic treatment with various doxorubicin based regimens and only 15 patients could be evaluated for treatment response: no conclusion was drawn on the predictive value of P-gp expression on treatment response. Furthermore, the P-gp expression, evaluated in primary STS specimens, was only an independent prognostic indicator when AJCC stages I and IV (with metastatic disease) patients were excluded. Their conclusion that P-gp expression is not only associated with chemosensitivity but also reflects aggressive tumor behavior, seems therefore limited to AJCC stages II and III STS. In our study in uniformly treated metastasized STS, no clear relation between P-gp expression, OS or PFS could be established. Other studies evaluating P-gp expression in STS also did not find a clear relationship with clinical outcome and even the reported predictive value in pediatric sarcomas is subject of debate [102,103,106]. Other immunohistological studies, using different monoclonal antibodies, revealed percentage of P-gp positive STS between 18% and 62% [100,104-106,301,302,324], as has been observed in our study in a heterogeneous group of malignant mesenchymal tumors. A correlation with tumor grade and tumor type was observed in some studies [100,297]. No correlation of P-gp with tumor grade or type could be found in the present study, which might at least partly be explained by the heterogeneity and the relatively small numbers of STS with different tumor grades.

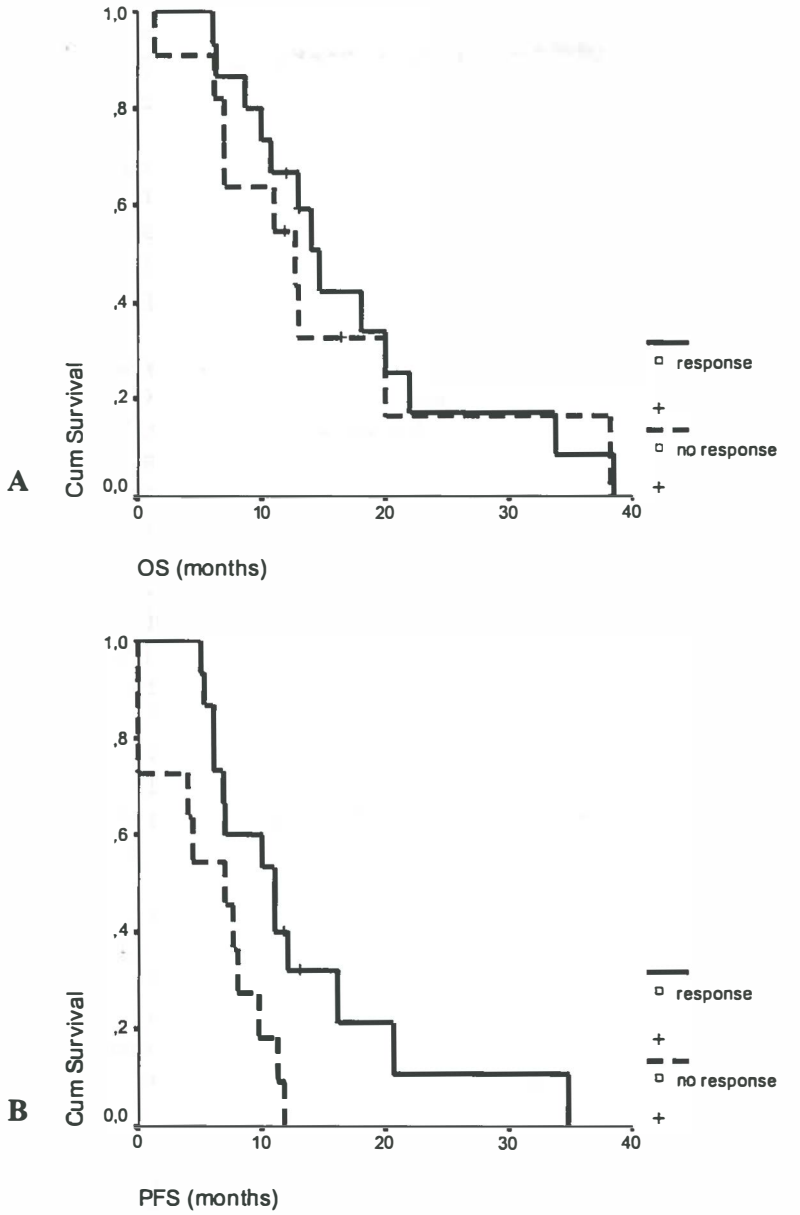
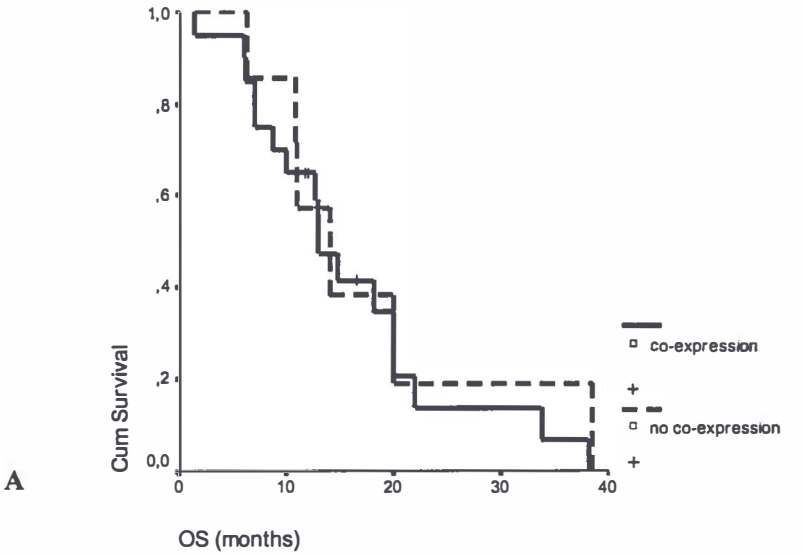


Figure 2
Overall survival (OS) (a) and progression free survival (PFS) (b) of the 27 sarcoma patients in relation to tumor response.



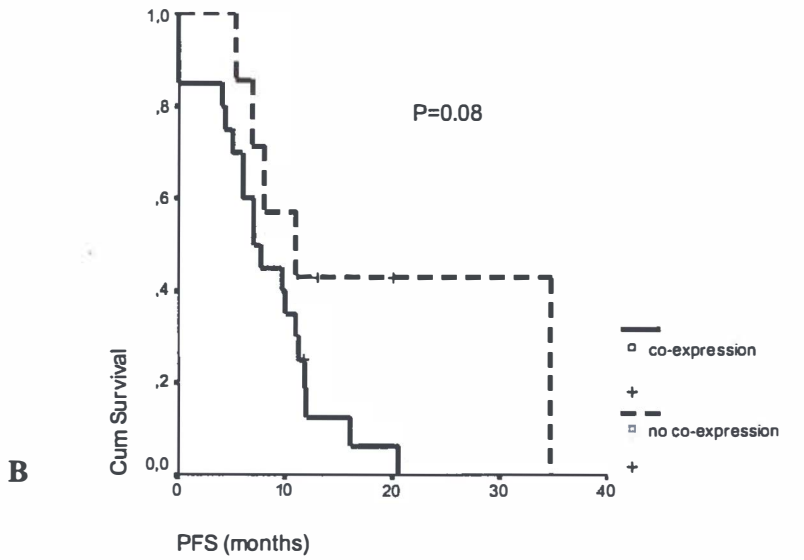


Figure 3
Overall survival (OS) (a) and progression free survival (PFS) (b) of the 27 sarcoma patients with tumors with and without co-expression of MDR proteins.

Furthermore, in STS it was shown that not all immunohistologically detected P-gp expressing tumors have functional P-gp *in vitro* [304]. Further studies evaluating the amount of MDR1 mRNA, P-gp expression and P-gp function simultaneously in a large series of STS, treated with the same chemotherapeutic regimen, are necessary to determine which of the techniques has the highest predictive value.

Much less is known about expression of the drug efflux pump MRP_1 in STS. In our group MRP_1 protein expression was detected in 64% of the STS and localized in predominantly the cytoplasm, although in the cases with moderate or strong staining also membranous staining was observed, as has been reported earlier [305]. Co-expression with P-gp was observed in 36% of the cases, which is highly comparable with the study of Oda et al. who detected MRP_1 mRNA in 56% of the STS and found co-expression with the MDR1 mRNA in 38% [108]. In the latter study a correlation of tumor grade with co-expression of MDR1 and MRP1 mRNA was also found, but we could not confirm such a relation, probably because of the heterogeneous small group of tumors in our study. Although not statistically significant, it should be noted that 9/11 (82%) of the cases with no response were MRP_1 positive and 9/16 (56%) of the responders were MRP_1 negative

No reports, so far, have been published on the expression of LRP in STS. In this study it was observed that 78% of the STS was LRP positive and was localized in the cytoplasm, as has been described before [96,292]. Expression of LRP was not correlated with P-gp or MRP_1, which is important since it has been reported that LRP is involved in doxorubicin and vincristine resistance and could therefore have an additional function in drug resistance to EVI [289]. However, the exact role of LRP in drug resistance remains not clear and it is thought that LRP is also involved in the transport of other cytotoxic agents than those involved in MDR mediated P-gp or MRP_1 [289,292,296,306].

The results of this study show that 93% of the STS has expression of one or more known drug resistance associated proteins. The assessment of histopath scores did not change the results of the various comparisons. This suggest that the percentage of MDR expressing tumor cells within a tumor is at least of equal importance as the intensity of protein expression within the tumor cells. However, when data concerning the studied proteins are limited, histopath scores provide valuable information regarding the relation between staining intensity and percentage of stained cells.

STS have a relatively high expression of all three MDR proteins. In other doxorubicin treated solid tumors, such as breast cancer, P-gp expression was observed in 10 - 50% of the cases, MRP_1 in 80% and LRP in 75% and no relation with response to chemotherapy was observed [313]. In ovarian cancer, in which P-gp was detected in 16-17% of the tumors, MRP_1 in 44-68% and LRP in 74-77%, the expression of LRP, but not P-gp or MRP_1, might have prognostic value [96,314]. In STS, the (co-)expression of P-gp, MRP_1 or LRP had no predictive value on the treatment response. Since there is still a favorable response rate of 59%, indicating that the EVI treatment has an encouraging effect on STS, the individual intact detoxifying mechanisms in the STS can only be a partial clarification for the 41% poor responses.

P-gp, MRP_1 and LRP was not of prognostic value for OS and PFS. A tumor response was associated with a better PFS but not with a better OS as has been observed earlier by Van Glabbeke et al in a large series of advanced STS [87]. When the co-expression of the drug resistance associated proteins was examined, regardless which two or three proteins were expressed, PFS was notably, though not significant (p=0.08), shorter for the patients with STS with co-expression as compared to the patients with STS without co-expression, although it did not affect OS. This finding might indicate that the expression of more than one MDR protein is more important than focusing on just one protein or mechanism, as has also been suggested by Izquierdo et al. [289]. In this way co-expression patterns might provide clinically useful information. The relevance of MRP2-5 and other drug resistance related proteins [354] and the possible relation with the apoptotic pathway [355-357] is of more speculation , but could be helpful in clarifying the importance of these proteins in clinical drug resistance.

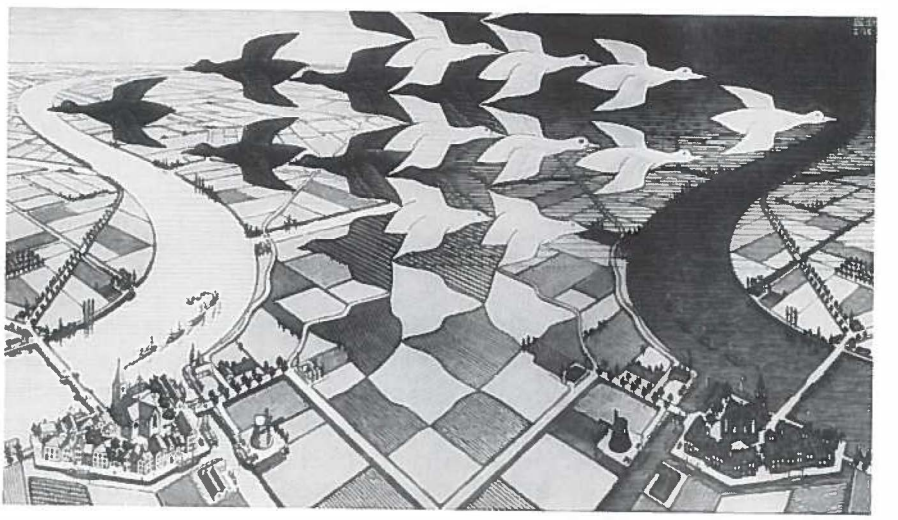
In conclusion, a high percentage of STS is expressing P-gp, MRP_1 or LRP, but the expression of these drug resistance associated proteins can not predict tumor response in the individual patient with a metastasized STS. Co-expression of two or more of the drug resistance associated proteins P-gp, MRP_1 and/or LRP showed a tendency to predict PFS. Therefore, future analyses should not focus on one MDR protein, but have to investigate the combined expression of mechanisms involved in drug resistance.

PART II

(CYTO)GENETICS

Chapter 10

COMPUTER ASSISTED CYTOGENETIC ANALYSIS OF 51 MALIGNANT PERIPHERAL NERVE SHEATH TUMORS: SPORADIC VERSUS NEUROFIBROMATOSIS TYPE 1 ASSOCIATED MALIGNANT SCHWANNOMAS



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SUMMARY

Cytogenetic studies in small groups of patients with malignant peripheral nerve sheath tumors (MPNST) revealed complex karyotypes with no consistent changes. A computer assisted cytogenetic analysis using a cytogenetic database was performed, to determine recurrent cytogenetic alterations in 51 MPNSTs (44 from the literature and 7 new cases) and to allow direct cytogenetic comparison between NF-1 associated and sporadic MPNSTs. Significant ($p<0.05$) loss was observed in the chromosomal regions 9p2, 11p1, 11q2 and 18p1. Also, loss in 1p3, 9p1, 11q1, 12q2, 17p1, 18q1-q2, 19p1, 22q1, X and Y was detected. Gain of chromosomal material was found in chromosome 7, especially 7q1 ($p<0.05$). Most involved breakpoints were: 1p13, 1q21, 7p22, 9p11, 17p11, 17q11, 22q11. Cytogenetic differences between NF-1 associated and sporadic MPNSTs included a relative loss of chromosomal material in NF-1 associated MPNSTs in 1p3, 4p1 and 21p1-q2 and a relative gain in 15p1-q1. Differences in breakpoints between the NF-1 associated and the sporadic MPNST group were observed in 1p21-22 (28% of NF-1 vs 0% of sporadic MPNSTs), 1p32-34 (17% vs 0%), 8p11-12 (7% vs 27%) and 17q10-12 (24% vs 7%). This approach, in which the cytogenetic results of various reports are combined, shows that losses in 9p2 and gains in 7q1 could be of oncogenetic importance in MPNSTs. Loss of 17q1, on which the NF-1 gene has been located (17q11.2), is not a common cytogenetic finding in NF-1 associated MPNSTs. The observed differences between NF-1 associated and sporadic MPNSTs might reflect different oncogenetic pathways.

INTRODUCTION

Cytogenetic analysis is helpful in clarifying oncogenesis of various malignant solid tumors and the observation of recurrent chromosome abnormalities led to the detection of tumor specific chromosomal alterations which are of diagnostic value [61,358-360]. Characteristic cytogenetic alterations have been found in several histologic types of soft tissue sarcomas (STS) [8,49,53]. About 5-10 % of the STS are malignant peripheral nerve sheath tumors (MPNSTs). They have also been referred to as malignant schwannoma, neurofibrosarcoma and neurogenic sarcoma. MPNSTs are highly aggressive neoplasms and half of them arise from neurofibromas in patients with neurofibromatosis type 1 (NF-1) or Von Recklinghausen's disease. About 10 % of all NF-1 patients develop a MPNST at a mean age of 30 years and the 5-year overall survival rate is 44 % [8,361]. Previous cytogenetic studies on MPNSTs revealed no consistent karyotypic pattern [49,362-374]. Since most karyotypes of MPNSTs are highly complex and difficult to interpret, studies focusing on common chromosome abnormalities are cumbersome. Studies in relatively small groups of MPNSTs reported near triploid or hypodiploid number of chromosomes and many structural rearrangements resulting in (partial) loss of chromosomal material of 1p, 9p, 11, 12p, 14q, 17q, 22q, X and Y or gain of chromosomes 2 and 7 [362,365-370, 373].

The neurofibromatosis-1 gene (NF-1-gene) is located on 17q11.2 and is thought to act as a tumor suppressor gene encoding for neurofibromin [375,376]. Some investigators hypothesized that the development of MPNSTs is a multistep process in which NF-1 mutations or loss of chromosome 17q is an early event in the tumorigenesis [366,367].
To determine meaningful cytogenetic changes observed in MPNSTs and discern possible cytogenetic patterns, a computer assisted cytogenetic analysis was performed on 46 MPNSTs reported in the literature and 7 new cases with an abnormal karyotype. A database was constructed which permits the detection of statistically significant non-random chromosomal aberrations and allows direct comparison of different karyotypes. This new approach was used to discover non-random changes in chromosomal material and to detect both frequently involved breakpoints in MPNSTs. Furthermore, this approach was used to analyze the cytogenetic differences between NF-1 associated MPNSTs and sporadic MPNSTs.

MATERIALS AND METHODS

A literature review using Medline® was performed to find reports of cytogenetic studies of MPNSTs and 44 cases, including 4 malignant triton tumors, with abnormal karyotypes were found [49,362-374]. The patients included 20 males and 24 females ranging in age from 2 to 82 years. Twenty-five patients were reported to have a history of neurofibromatosis type-1 and of nine patients the NF-1 status was unclear. In most cases S100 immunohistochemistry was used to support the histological diagnosis in the non-NF1 MPNSTs (*table 1*). Fifteen cases had an incomplete karyotype according to the ISCN '95 [377].

In our institute tumor material was obtained for cytogenetic analysis from 7 patients with a MPNST. Diagnosis was made according to the criteria described by Enzinger and Weiss including immunohistochemical detection of the S100 protein [8]. A cytogenetic analysis of all specimens was carried out according to standard procedures and the karyotypes were described according to the ISCN 1995 Guidelines for Cancer [377]. A summary of the clinical and histopathological data of these cases is presented in *table 2*. Case 1 is an outside case of which only suspension was available and diagnostic criteria could not be identified retropectively.

The analyzed group thus consisted of 28 NF-1 associated MPNSTs, 14 sporadic MPNSTs and 9 MPNSTs with an unknown NF-1-status and there were 35 primary tumors (27 with a complete karyotype), 4 metastases (2 with a complete karyotype), 1 local recurrence (with a complete karyotype) and 11 MPNSTs of which the tumor status was unclear (7 with a complete karyotype). If more than one tumor per patient was described, it was decided to use the karyotype of one tumor i.e. the primary tumor (if possible) to avoid overrepresentation.

A *database* was constructed using dBase V Windows and consisted of four main parts related to the described karyotype: 1) the patient data and histological data of the tumor, 2) the gain and loss of chromosomal material, 3) the breakpoints per chromosome arm, and 4) the structural rearrangements. After interpretation of the

karyotype, the gains and losses of chromosomal material were entered. In short, each chromosome was divided according to the ideogram at 400 bands level as described by the ISCN, in such a way that net gains and losses in 1p11-p13 were summarized in 1p1, 1p21-22 was summarized in 1p2, etc. . In case of (net) loss in these parts of one of the chromosomes a -1 was entered. If the same parts were lost in two chromosomes a -2 was entered. Similarly, if (net) gain occurred in one of these chromosomal parts a +1 was entered, etc. . Only changes as compared to the constitutional karyotype were evaluated. In this way 86 chromosomal regions per karyotype were recorded. All structural rearrangements were categorized per type of structural chromosome rearrangement (according to the ISCN). Microsoft Excel 5.0a Windows was used to transform the gains and losses of chromosomal material of all non-diploid karyotypes to diploid karyotypes. Before this diploidization, the changes in the chromosomal material of the X-chromosome of men were multiplied by 2 in order to compare male with female karyotypes.

To analyze the data, Microsoft Excel 5.0a Windows was further used to compute: the number of tumors with gains or losses in a particular chromosomal region, the mean change in chromosomal material per chromosomal region and the mean number of breakpoints per chromosome arm. Mean change in chromosomal material in each of the 86 chromosomal regions is expressed as a chromosomal change ratio (CCR) which is defined as the absolute change in a specific chromosomal region as compared to a normal diploid karyotype e.g. if both chromosomes 1 of all analyzed tumors are lost, the mean change in the specific chromosomal regions 1p1,1p2,1p3, 1q1,1q2,1q3 and 1q4 is: -2.00 in each of the regions. Only full abnormal karyotypes were used for the analysis of the over- or underrepresentation of chromosomes or chromosomal material. Reports that included incomplete karyotypes, which only described the clonal numerical and structural rearrangements, were only included in the breakpoint analysis. Graphs were constructed to visualize the change in chromosomal material, the amount of breakpoints per chromosome arm and the differences between selected groups, i.e. the NF-1 associated MPNSTs versus the sporadic MPNSTs.

To identify chromosomal regions with significant gains or losses, 95% confidence intervals (CI) were calculated using Microsoft Excel 5.0a Windows. To avoid the introduction of significant-appearing associations by chance and to address the problem of multiple comparison, the significance level of 0.05 was adjusted downwards. The number of tests performed is 86. The tests are however dependent, because deletions take place for chromosomal segments in several tests. Therefore a Bonferroni correction factor of 86 is conservative. A mean number of breakpoints in each chromosome arm > 3.1 SD was considered to be significant reflecting a probability threshold of 0.1. As an overall test whether the differences in amount of chromosomal material for each chromosomal region and the number of breakpoints between NF-1 associated and sporadic MPNSTs were statistically significant, an analysis of variance was performed where the statistical interaction between the chromosomal position x tumor type was calculated. The calculations were made on the residuals after adjusting for person differences in amount of chromosomal

material. Systat 5.1 and 6.0 for Windows was used for the calculation. A p-value <0.05 was considered statistically significant.

Table 1. Diagnostic criteria for the 44 MPNST utilised by the different authors.

author	number of MPNST (n)	with neuro-fibromatosis-1 (n)	S100 staining	peripheral nerve association
Becher *et al.*, 1984 *	2	1	ND	uncertain
Riccardi *et al.*, 1986	4	4	ND	yes
Decker *et al.*, 1990	1	1	ND	uncertain
Fletcher *et al.*, 1991 *	8	?	ND	not reported
Glover *et al.*, 1991	1	1	ND	not reported
Rey *et al.*, 1993	1	1	ND	uncertain
Jhanwar *et al.*, 1994	10	9	+	not reported
Orndal *et al.*, 1994	1	1	+	not reported
Travis *et al.*, 1994	2	0	+	not reported
Mertens *et al.*, 1995	1	?	+	not reported
Mertens *et al.*, 1995	7	4	+	not reported
Sciot *et al.*, 1995	1	?	+	not reported
McComb *et al.*, 1996	2	2	ND	yes
Rao *et al.*, 1996	3	1	+	yes

ND: not done, +: done in (part of) the reported cases, * the karyotypes of the MPNST reported by Becher *et al.*, 1984 and Fletcher *et al.*, 1991 are described in detail by A.A. Sandberg in *The chromosomes in human cancer and leukemia,* Elsevier, New York (1990).

RESULTS

Nearly all cases showed a complex karyotype consisting of many numerical and structural rearrangements. The median chromosome number was 58 (range 34 - 270 chromosomes).

Change in chromosomal material per chromosomal region (Fig. 1):

Construction of 95% CI revealed significant loss, expressed as chromosomal change ratio (CCR), of chromosomal material in the chromosomal regions 9p2 (-0.64 CCR), 11p1 (-0.27 CCR), 11q2 (-0.32 CCR) and 18p1(-0.34). Although not statistically significant, there was also notable loss in 1p3, 9p1, 11q1, 12q2, 13p1, 14p1, 17p1, 18q1-q2, 19p1, 22q1, X and Y. A statistically significant gain of chromosomal material was observed in the 7q1 region (+0.42 CCR) , whereas a notable gain was observed in all regions of chromosome 7.

Table 2. Summary of clinical and cytogenetic data of the 7 patients with MPNSTs not reported earlier.

Case	Age/ sex	Location	NF-1	Karyotype
1	66/M	? / ?	no	36~43,XY,der(1)t(1;?;3)(p13;?;p24),-2,add(3)(p21),+der(4)t(2;4)(p11;q12),-6,-7,del(7)(q11), i(8)(q10),-9,dic(9;11)(p11;p11), der(10)t(10;17)(q22;q11),add(12)(p11),-13,-17,-17,-18,-21, +1~5 mar [cp9]
2a	41/M	sciatic nerve primary tumor	no	36~38,X,-Y,add(1)(q21),add(3)(q10),-5,-6,add(7)(q36),-8,-8,-9,der(9)add(9)(p12)t(1;9) (q23;q22),-10,add(11)(p10), add(12)(q22), -13,-13,-14,-15,-16,-16,-17,-17,-18, dic(18;?;18) (q21;?;p11),-19, -20,-21,-22,-22,+mar1,+mar2, +mar3, +mar4,+mar5,+4~9mar[cp5] / 67~72,idem x2, -add(3)(q10),-mar2,-mar5, +6~17mar[cp3]
2b		lung metastasis	no	40,X-Y,add(1)(q21),add(3)(p22),-6,der(7)t(7;?;9)(q32;?;q13), add(8)(q24),?add(8)(p11),-9,-9,-10, add(11)(p15), der(11)t(11;?;12)(p11;?;q15),-12,-13,add(15)(q22),-16, del(16)(p12), -17,-17,-18,add(18)(q23),-19,-20,-21,+22, +del(22)(q12),+der(?)t(?;1)(?;q25),+r,+mar1,+mar2, +mar3, +mar4[cp2]/66~79,idemx2,-22,-r,-mar3,-mar4,-mar4[cp5] /143,idemx4,-mar2x4,-mar3x3, mar4x4[1]/46,XY[2]
3	52/F	mediastinum	yes	42~46,XX,t(1;17)(q25;q12)[cp2]
4	32/F	right thigh	no	67~73,X,-X,der(X)t(X;?;9)(q11;?;q12),der(1)t(1;15)(p13;q15), +der(1)t(1;15)(p13;q15),+2,-3, add(3)(q25),+der(4;6)(p10;p10), del(6)(p22p24),+5,-6,-6,+7,+8,+add(8)(p23),-9,-inv(9) (p11q13)c, der(9)t(9;9)(p21;q13),-10,+10,-11,-12,der(12)t(4;12)(q11;p11), dic(12;22)(p11;q10),-13,-14,-15,-15,add(15)(p11),-16, add(16)(q11), -17, -18,+18,-19,der(19)t(17;19) (q12;q13.4),-20,-20, +21,+22,+der(?)t(?;14)(?;q11),+r1,+r2,+1~7mar[cp10] /46,XX,inv(9)(p11q13)c[1]
5	62/F	right axilla	no	54~61,X,+X,Y,+der(1;8)(q10;q10),+der(1;14)(p13;q11),+add(2)(q11)x2,+3,+add(3)(q11),+der(3)t(3;14)(q1?2;q11), +4, +?add(4)(q11),+5,+5,+add(7)(q36)x2,+add(7)(q36)x2, +der(7)del(7)(p15p21)del(7)(q11.1q21)x2,+add(8)(p11), +add(8)(p2?1),der(9)t(9;?;15)(p21;?;q11),der(9)t(9;?;15) (p21;?;q11),-12,-12,der(12)t(7;12)(q?21q?11)x2,-13, dup(13)(q12q34)x2,-14,-14,i(14)(q10),-15,-15,der(16)t(5;16) (q15;p13)x2,-17,-17,+psudic(18;12)(p11.3;p11.2)x2, +r(19)(p13;q13),+2r, 2~3dmin[cp10]
6	53/M	interthora-coscapular	yes	65~70,XY,-X,add(1)(p22),add(1)(q21),-3,-4,-4,-4,add(5)(q11),+add(5)(q11),der(6)add(6)(p22) idic(6)(q14), +7,+i(7)(p10),-8,-8,-9,der(9)t(4;9)(q12;p24)x2,-10,-11,-11,+13, -14, add(15) (q25),-17,-18,-19,add(19)(p13),-20, der(20)t(1;20)(q25;q13),-21,+der(?)t(2;?;8)(p11;?;q13), +der(?)t(?;8)(?;q13),+mar1,+mar2x5 [cp9]
7	25/F*	neck	yes	59~72,X,-X,add(X)(q23),-1,add(1)(p11),add(1)(p23), del(1)(q21),der(1)t(1;22)(p13;q11), add(2)(q21),add(2)(q31), +del(2)(q14~q21),+3,-4,-4,-4,+5,der(6)t(6;17)(p11;q21), +der(6)t(6;17)(p11;q21),+add(7)(p22)x2,+add(8)(p11),+i(8)(q10), +der(9)t(1;9)(q12;p13),-10,add(10)(p12),+add(10)(p12),-11, der(12)t(4;12)(q13;p12),+der(12)t(4;12)(q13;p12),+13,-14,-14, +15, +15,+add(16)(q23),-17,-17,+18,-19,-20,add(20)(p12),-21, -21,-22,-22,-22, +der(?)t(?;1)(?;p22),+1~5mar[cp11]

* patient with malignant triton tumor

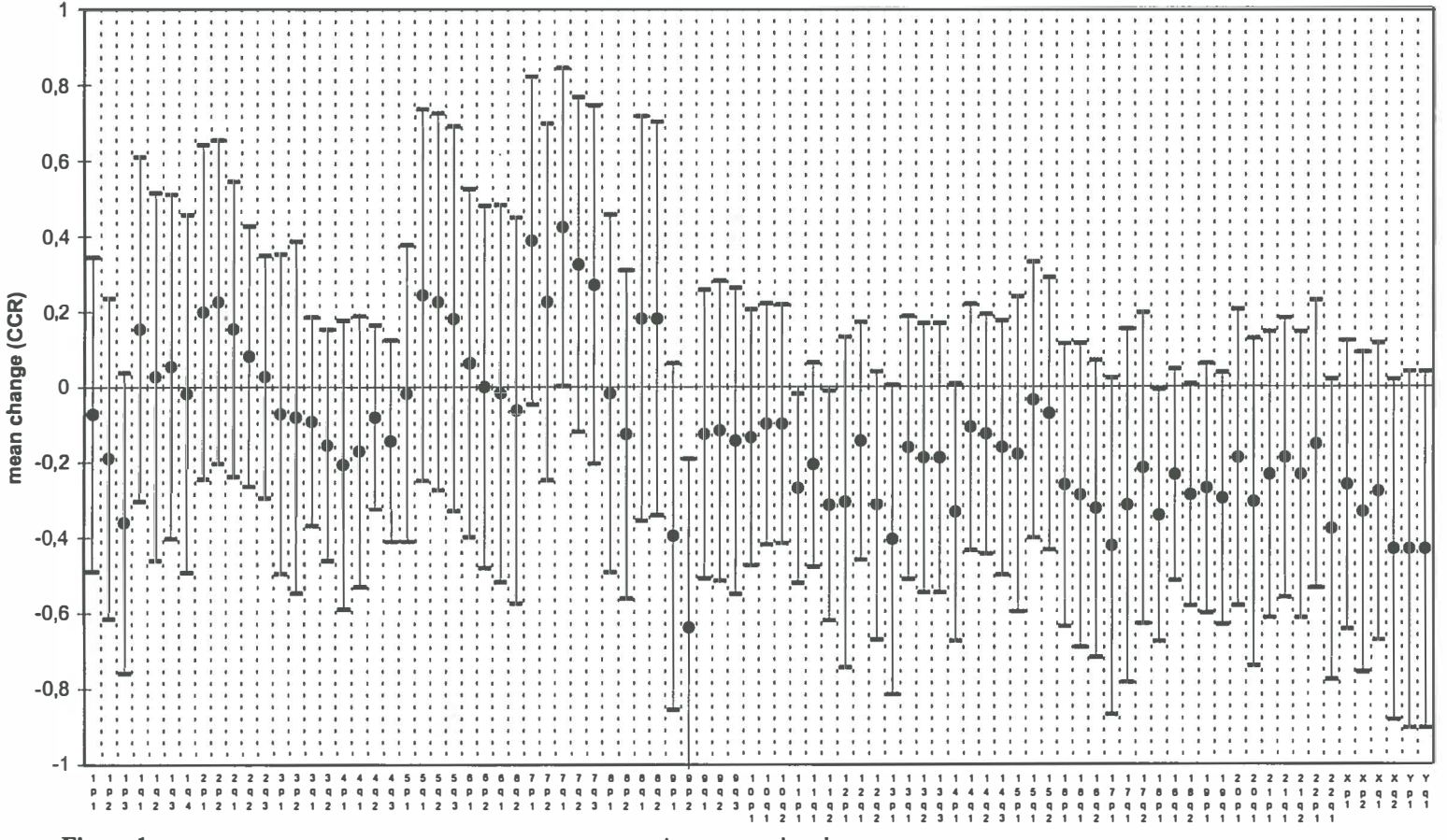


Figure 1
95% confidence intervals of the change in chromosomal material in the 86 chromosomal regions after computer assisted cytogenetic analysis of all 37 MPNSTs with a complete karyotype

Breakpoints:

Fifty-one percent of the tumors had breakpoints in 1p and in 39% in 1q. A notable percentage of the MPNSTs had breakpoints in 7p, 9p, 17q and 22q (33%, 39%, 29% and 29%, respectively). The total number of breakpoints per chromosome arm varied from 0 to 32 (median: 9). There were no chromosome arms with a statistically significant high amount of breakpoints. In 1p, 1q, 7p, 9p, 17q and 22q the mean number of breakpoints was relatively high (Fig. 2). Most involved breakpoints were: 1p13 (18% of the tumors), 1q21(10%), 7p22 (18%), 9p11 (12%), 17q11 (14%), 22q11 (14%).

NF-1 associated versus sporadic MPNSTs

When the group of NF-1 associated MPNSTs (n = 17) was compared with the sporadic MPNSTs (n = 11) a notable (but not statistically significant) difference was found in chromosomal regions 1p3, 4p1, 15p1-q1, 21p1-q2 (Fig. 3). Relative loss of chromosomal material in NF-1 associated MPNSTs as compared to sporadic MPNSTs was found in 1p3, 4p1, 21p1-q2. Relative loss of chromosomal material in sporadic MPNSTs as compared to NF-1 associated MPNSTs was noticed in 15p1-q1. Shared chromosomal loss was found in chromosomes 9, 11, 17, 18, 19, 20 and Y as well as in 14p1. Shared gain was observed in chromosome 7.

Differences in breakpoints between the NF-1 associated and the sporadic MPNSTs were observed in 1p, 8p and 17q (Fig. 4). Mean number of breakpoints per tumor in 1p was 0.82 for NF-1 associated MPNSTs and 0.43 for sporadic MPNSTs, whereas these amounts were 0.07 and 0.43 for 8p and 0.43 and 0.07 for 17q, respectively. Breakpoints in 1p21-22 were found in 29% (8/28) of the NF-1 associated tumors and in none (0/14) of the sporadic MPNSTs. Other frequently involved breakpoint regions differing between both groups were: 1p32-34 (in 18% of the NF-1 MPNSTs and in none of the sporadic MPNSTs), 8p11-12 (in 7% of the NF-1 MPNSTs and in 29% of the sporadic MPNSTs), 17q10-12 (25% of the NF-1 associated MPNSTs and in 7% of the sporadic MPNSTs). An equal amount of breakpoints was observed in 1q (mean number of breakpoints per tumor: 0.54 in the NF-1 associated MPNSTs group and 0.50 in the sporadic MPNSTs group), 9p (0.36 and 0.29, respectively) and 12p (0.29 and 0. 21, respectively).

DISCUSSION

Cytogenetic analysis has proven to be an important tool in the detection of tumor specific alterations in the human genome [46-49]. This has been achieved by the karyotypic analysis of high numbers of tumors in which recurrent chromosomal alterations appeared to be related to the formation of an oncogene or the deletion of a functional tumor suppressor gene. This resulted in the detection of genes which either may be characteristic for a tumor type or may be involved in cell proliferation in general [50-52]. It is difficult to ascertain whether a recurrent chromosomal anomaly is involved in the primary steps of the oncogenetic process or just a relatively late

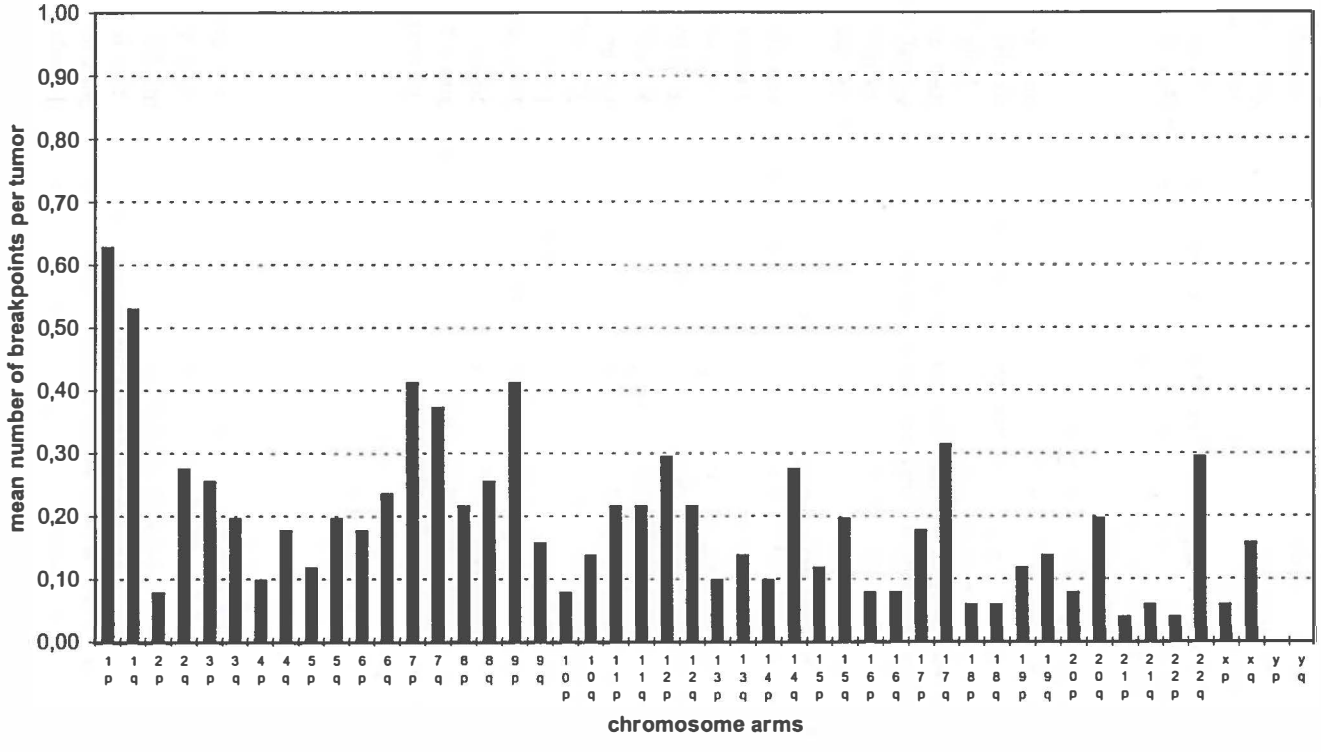


Figure 2
Mean number of breakpoints per tumor in each of the chromosome arms after computer assisted cytogenetic analysis of 51 MPNSTs.

footprint. Especially complex karyotypes of solid tumors can be difficult to relate to the process of oncogenesis because of the amount of chromosomal abnormalities which may all be of certain importance. Therefore, the analysis of high numbers of tumors is of utmost importance. The next step is to directly compare all the chromosomal alterations in different groups of tumors e.g. primary tumors and metastases. However, most of the published studies presented karyotypes in such a way that even shared chromosomal alterations are difficult to detect. A few studies visualized the information of karyotypes in graphics or transformed the cytogenetic information in such a way that statistical analysis was possible, although a lot of cytogenetic information could not be used in this way or a direct comparison was not possible [77,378,379]. This problem of interpreting and comparing different tumor groups was the reason for the construction of the database which was used in this study. Since all karyotypes are interpreted and entered into this database uniformly, this computer assisted approach allows the direct comparison of specific tumor groups. This direct comparison of cytogenetic data and the visualization in so called karyographs makes it easier to interpret the differences between groups of tumors. Furthermore, it is possible to select those tumors with a specific structural rearrangement or in which specific breakpoints are involved and combine them with other databases in which, for instance, treatment response or survival data are logged. This way of analysis can not be a replacement of conventional cytogenetic analysis, since it is only a tool to combine various cytogenetic and other data into a more accessible collection of information after the interpretation of the karyotypes. This results in loss of cytogenetically derived information, since all karyotypes are forced to be transformed into a uniformly created set of data. Especially, problems arise when interpreting and transforming composition karyotypes in which reported chromosomal abnormalities can be conflicting i.e. both loss and gain of a chromosome 1 can be described in one composition karyotype. In these exceptional cases, it was decided to interpret these abnormalities as no change in chromosomal material, since these changes, resulting in gain of chromosomes in one tumor cell or clone and a loss in another within the same tumor, are most likely not the hallmarks in the oncogenetic process. Furthermore, one has to consider the differences in cytogenetic nomenclature which have emerged during the last decades, when cytogenetic studies of uncommon solid tumors are interpreted. Although structural abnormalities and the number of marker chromosomes are entered into the database, the marker chromosomes themselves can not be included in the analysis of over- and underrepresentation of chromosomal material.

This method of computer assisted cytogenetic analysis gives the opportunity to expand the understanding of the cytogenetic anomalies of uncommon solid tumors by taken together all conventional cytogenetic studies of small numbers of cases and extract meaningful shared cytogenetic aberrations. The combination of conventional cytogenetic analysis used in this study together with techniques like comparative genomic hybridization, spectral karyotyping and multicolor fluorescence in situ hybridization, might detect substantial chromosomal alterations of diagnostic, oncogenetic or prognostic importance.

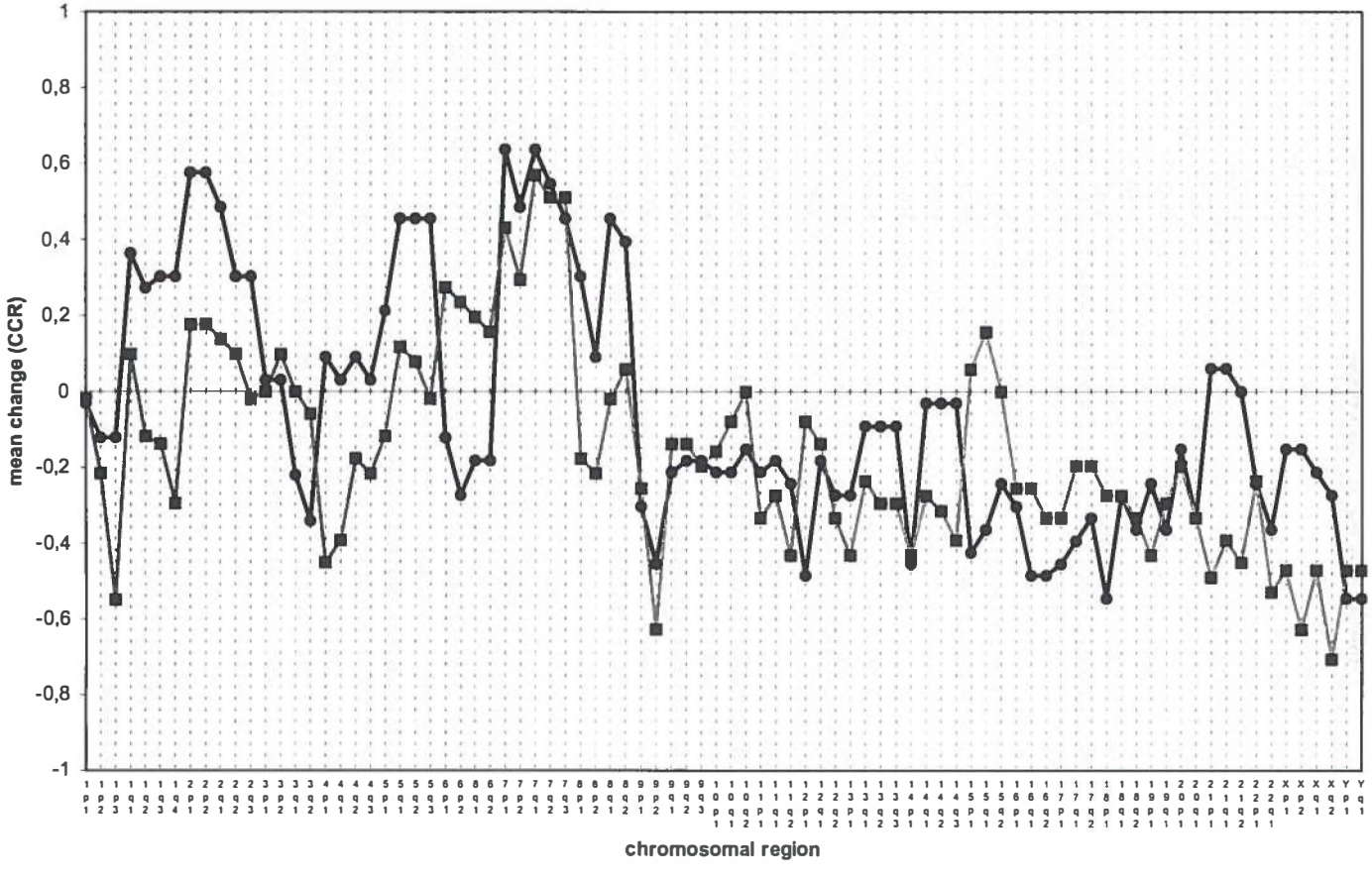


Figure 3
Mean change in chromosomal material in the 86 chromosomal regions after computer assisted cytogenetic analysis of 11 sporadic MPNSTs (●, black line) and 17 NF-1 associated MPNSTs (■, grey line) with a complete karyotype.

In this study, a series of 51 MPNSTs of both NF-1 patients and non-NF-1 patients was analyzed using this computer assisted approach. Since the objective of this approach was to detect the most common cytogenetic abnormalities in a large number of MPNSTs, it was decided to use karyotypes of primary MPNSTs as well as metastatic MPNSTs and tumors of which the tumor status was not clearly described. After interpretation of the karyotypes as reported in the literature and our own cases, all karyotypes were transformed and entered into the database. In this way the changes in chromosomal material in each of 86 previously determined chromosomal regions were analyzed. In most tumors the change in chromosomal material was not clearly confined to one region: the number of tumors with a normal amount of chromosomal material in each of the 86 chromosomal regions was high. If a change in chromosomal material was observed, the majority of tumors had loss of chromosomal material and this was observed in virtually all the chromosomal regions.

It was found that the loss in 9p, 11 and 18, as reported in several individual reports, was restricted to statistically significant loss in 9p2, 11p1, 11q2 and 18p1[365-367,369,380]. Since a lot of genes are located on these particular regions, no well argumented suggestion of the involved genes can be made. However, recent studies have shown that many genes located on 9p2 are involved in cell cycle control and proliferation like CDKN2A and CDKN2B encoding for p16 [381], whereas on the chromosomal regions 11p1 CDKN1C encoding for p57 is mapped [382]. Very recently, Lothe et al. (personal communication, 1998) analyzed 12 MPNSTs by FISH and allelic imbalance studies with 9p21-23 markers. They found interstitial deletions that supported CDKN2A as a possible target gene that might contribute to the development of MPNSTs. The earlier reported loss of sex chromosome material was also observed in this study, but was not statistically significant [366,370]. The reported alterations in 12p, 14q, 17q and 22q can not be confirmed in this analysis [362,366,367,370,373]. Both gains and losses were found in these chromosomal regions, leading to a balanced net result.

Gain of chromosomal material in chromosome 7 was observed in 17 of the 37 MPNSTs. Analyzing the change in chromosomal material of all MPNSTs, mean gain of chromosomal material in 7p and 7q varied between +0.42 CCR and +0.47 CCR . Several other studies reported gain of chromosome 7 material in MPNSTs [363,366,370,372]. The observed gain of chromosomal material was most manifest in the 7q1 region on which genes like p19A, associated with cell cycle regulation, are mapped [383]. Further studies focusing on p19A and 7q1 may clarify the significance of this region in MPNSTs. In a recent study in neurofibromas of NF-1 patients the authors found multiple microsatellite alterations on chromosome 9 and concluded that chromosome 9 might have a role in the pathogenesis of neurofibromas [384]. An earlier study of 10 MPNSTs using comparative genomic hybridization, showed loss of 9p as well [380]. In our study the MPNSTs of both NF-1 patients and patients without signs of NF-1 had loss of 9p. These findings suggest that 9p alterations could be early events in the development of neurofibromas and subsequent transformation into a MPNST.

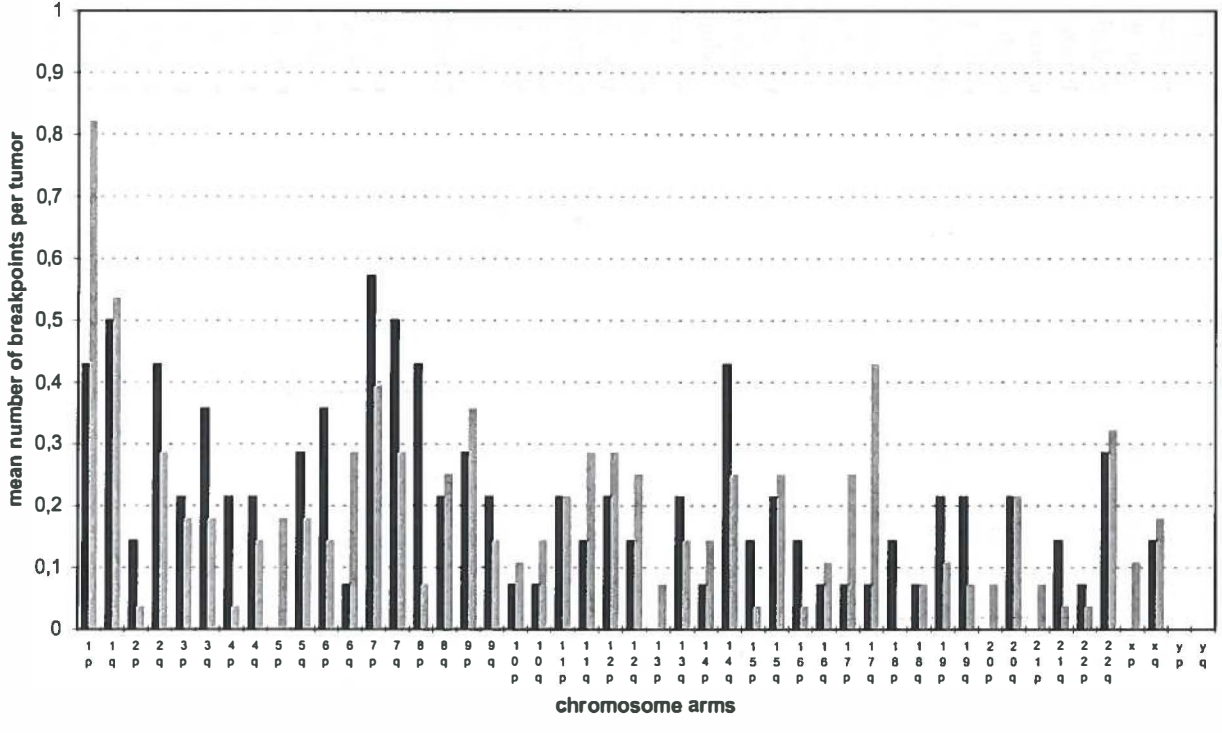


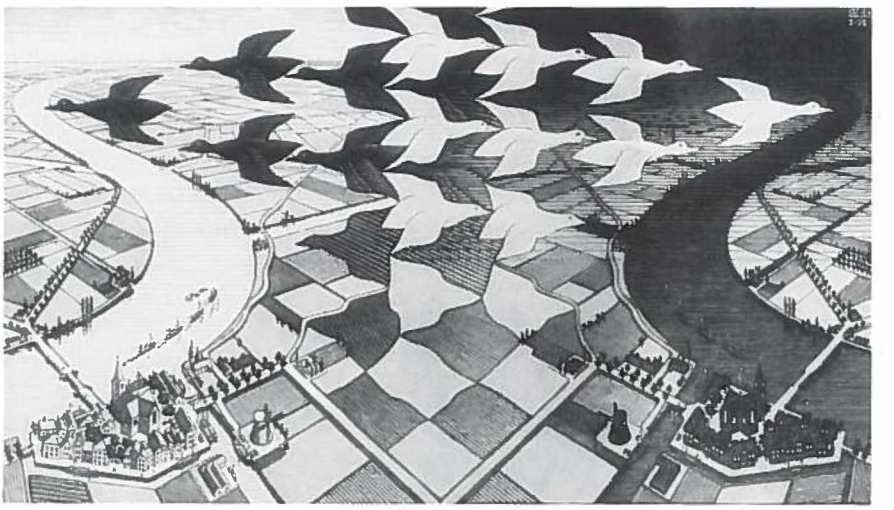
Figure 4
Mean number of breakpoints per tumor in each of the chromosome arms after computer assisted cytogenetic analysis of 14 sporadic MPNSTs (presented in black) and 28 NF-1 associated MPNSTs (presented in grey).

Breakpoint analysis of all MPNSTs did not reveal a characteristic breakpoint. The most involved breakpoints were 1p13 (18% of the tumors) and 7p22 (18% of the tumors). On 17q11.2 the NF-1 gene has been located. Although NF-1 gene alterations are present in the majority of NF-1 patients, cytogenetic loss of 17q11 is only present in 14 out of the 37 analyzed MPNSTs in this study. Breakpoints in 17q11 were only present in 14% all MPNSTs, whereas 55% of the MPNSTs were derived from NF-1 patients. However, differences between the NF-1 associated and sporadic MPNSTs were observed in the breakpoint analysis, since breakpoints in 17q10-12 were found in 25% of the NF-1 associated MPNSTs and in only 7% of the sporadic MPNSTs. Whether the observed differences between the two groups as found in the breakpoint analysis are relevant, has to be clarified in further studies. When the NF-1 associated MPNSTs were compared to the sporadic MPNSTs, the same pattern of gain in chromosome 7 and loss in chromosome 9p was noticed, indicating a common oncogenetic pathway. Loss of chromosomal material in 1p3, 4p1, 21p1-q2 appeared to be more frequent in NF-1 associated MPNSTs, whereas loss of 15p1-q1 appeared to be more specific for sporadic MPNSTs. Whether the differences are correlated with neurofibromatosis or not has to be elucidated. The earlier reported gain of 17q2 in NF-1 associated MPNSTs, detected by comparative genomic hybridization, could not be confirmed [380]. On 22q12.2 the NF-2 gene is located and it is suggested that loss of 22 is associated with the development of a MPNST, but in this study no significant losses of chromosomal material involving 22 or 22q1 were found [362].

In conclusion , this computer assisted approach has proven to be valuable in the analysis of large groups of karyotypes and can automatically detect common chromosomal alterations without bias caused by focusing on specific chromosomal abnormalities. This study did not unveil specific structural cytogenetic abnormalities, which could be of diagnostic importance in MPNSTs. However, the finding of a complex karyotype showing loss of 9p2 in combination with gain in 7q1 in a tumor histologically resembling a MPNST, could be of diagnostic importance. This approach revealed indications for cytogenetic differences between NF-1 associated and sporadic MPNSTs in chromosomal regions 1p3, 4p1, 21p1-q2 and 15p1-q1. Additional studies, combining different cytogenetic based techniques, might detect differences between NF-1 associated and sporadic MPNSTs which could be helpful in clarifying the oncogenesis of MPNSTs.

Chapter 11

META-ANALYSIS OF CYTOGENETIC FINDINGS IN GASTROINTESTINAL MESENCHYMAL TUMORS ("GIST"), SOFT TISSUE LEIOMYOSARCOMAS AND MALIGNANT PERIPHERAL NERVE SHEATH TUMORS



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SUMMARY

The histogenesis, oncogenesis and clinical behavior of mesenchymal tumors of the gastrointestinal tract are the subject of dispute, since these neoplasms may reveal either smooth muscle differentiation, resembling leiomyosarcomas of soft tissue (LMS), or neural differentiation, resembling malignant peripheral nerve sheath tumors (MPNST). Most of them are currently designated gastrointestinal stromal tumor (GIST). Previous cytogenetic studies did not clearly differentiate between GIST, LMS and MPNST. In this study, a computer assisted method of direct cytogenetic comparison was used to discover tumor specific chromosomal patterns in 16 gastrointestinal mesenchymal tumors ("GIST") and 14 LMS and 37 MPNST of soft tissue obtained from the literature and from our own data. In "GIST" a statistically significant loss was observed in chromosomal regions 13q2-q3, 14p1-14q2, 18p1-18q2, 22p1 and 22q1. In LMS the chromosomal changes did not reach statistical significance. However, important loss was found in 5p1, 14p1 and of chromosome 22, whereas gain was observed of chromosome X. In MPNST a significant loss of chromosomal material was detected in 9p2, 11p1, 11q2 and 18p1, as well as important loss in 22q; a significant gain was noticed in the 7q1 region. Breakpoint analysis revealed differences between "GIST" and LMS in 1p, 7p, 9q and 14. Differences in breakpoints between the "GIST" and MPNST were observed in 1q, 7q, 9 and 12. This cytogenetic meta-analysis supports the hypothesis that "GIST" is a distinct entity which differs from LMS and MPNST and is characterized by losses in 13q2-q3, 14p1-q2. However, the shared loss of chromosome 22 in "GIST", LMS and MPNST, might be indicative for a common oncogenetic pathway, whereas gain of 7q1 and loss of 18p1-q2 seems to be more associated with neural differentiation in MPNST and "GIST".

INTRODUCTION

Tumors of mesenchymal origin with features of smooth muscle differentiation may originate from various anatomic sites, i.e. the soft tissue, the uterus and the gastrointestinal tract. Leiomyosarcomas of soft tissue (LMS) account for approximately 10% of all soft tissue sarcomas and predominantly arise in the mesenchymal tissues of the extremities, the skin and the retroperitoneum [8]. Mesenchymal tumors of the gastrointestinal tract may have ultrastructural and immunohistochemical characteristics consistent with smooth muscle differentiation and these have in the past been referred to as LMS of the digestive tract. However, the histogenesis and oncogenesis of these tumors are the subject of dispute, since they may have features not only of smooth muscle differentiation, resembling LMS, but also of neural differentiation, resembling malignant peripheral nerve sheath tumors (MPNST) [307,385-388]. Mesenchymal tumors of the gastrointestinal tract now are collectively designated gastrointestinal stromal tumors (GIST) and are assumed to arise from interstitial cells of Cajal, to express CD34 and C-kit protein and to have

mutations in the C-kit gene [307,309,315,327,389]. Clinical outcome in GIST, LMS and MPNST is closely related to the presence of metastatic disease, which may require chemotherapeutic treatment. Chemotherapeutic studies report very poor responses in LMS, but do not clearly differentiate between metastatic soft tissue LMS or metastatic GIST [87,142,285,286,320].

Cytogenetic studies have revealed characteristic chromosomal alterations in various histologic types of soft tissue sarcomas [49,53,162,390]. However, cytogenetic analyses in "smooth muscle" neoplasms have mainly been performed in uterine smooth muscle tumors and cytogenetics in "leiomyosarcomas" did not clearly discriminate between GIST and soft tissue LMS. Consequently, the findings of monosomy 1, 9, 18 and 22 as well as the chromosomal changes in 1p13 observed in groups consisting of both LMS of the soft tissue and of the gastrointestinal tract have to be interpreted with caution [391,392]. In cytogenetic studies on mesenchymal tumors of the gastrointestinal tract, losses of chromosomes 14, 15, 18 and 22 were observed [393-395]. Comparative genomic hybridization revealed losses in 14q, 15 and 22 as well as gains in 3q, 5p, 8q, 17q and 19q but could not detect frequently involved breakpoints [396-398].

In order to compare complex karyotypes of different groups of tumors, we recently developed a computer assisted approach, with which the pattern of chromosomal change and the presence of frequently involved breakpoints can be evaluated [399]. In the current study this method was used to compare mesenchymal tumors of the gastrointestinal tract with LMS and MPNST of the soft tissue in an attempt to discover common and specific chromosomal changes in these groups.

MATERIALS AND METHODS

Patients and cytogenetic data

Twelve cases were diagnosed and cytogenetically analyzed in our institute. A histological diagnosis was made according to the criteria described by Enzinger and Weiss [8]for soft tissue tumors and according to Suster [307] for GIST. A cytogenetic analysis of all specimens was carried out according to standard procedures. The karyotypes were described according to the ISCN 1995 Guidelines for Cancer [377]. Eight cases (1 LMS and 7 MPNST of soft tissue) were previously published [53,399] . A summary of the clinical, histopathological and cytogenetic data of the 4 other cases is presented in Table 1.

Fifty-five cases with abnormal karyotypes were retrieved from the literature using Medline®, i.e. 15 cases of GIST or LMS of the digestive tract, 10 cases of soft tissue LMS and 30 of soft tissue MPNST 49,53,363-371,374,391-393,395,400-405. All cases had a complete karyotype description according to the ISCN '95 377.

The analyzed group thus consisted of 67 cases. Since cases described in the older literature as LMS of the gastrointestinal tract presumably include GIST, all gastrointestinal mesenchymal tumors were grouped together in a group designated "GIST". This consisted of 8 males and 8 females ranging in age from 46 to 83 years

(mean: 59 years, median: 56 years). The soft tissue LMS group consisted of 4 female and 10 male patients ranging in age from 29 to 79 years (mean: 54 years; median: 50 years). One study did not report the age of a male patient with a LMS [392]. In the MPNST group there were 20 male patients and 17 female patients ranging in age from 2 to 82 (mean: 39 years, median: 33 years). There were 38 primary tumors (5 "GIST", 6 LMS, 27 MPNST), 10 local recurrences (6 "GIST", 3 LMS, 1MPNST) and 3 distant metastases (1 LMS, 2 MPNST). In 16 cases (5 "GIST", 4 LMS, 7 MPNST) the tumor status was unclear. If more than one tumor per patient was described, it was decided to use the karyotype of one tumor, preferentially the primary tumor, to avoid overrepresentation.

Computer assisted comparison

A database was constructed using dBase V Windows and consisted of four main parts related to the described karyotype: 1) the patient data and histological data of the tumor, 2) the gain and loss of chromosomal material, 3) the breakpoints per chromosome arm, and 4) the structural rearrangements. After interpretation of the karyotype, the gains and losses of chromosomal material were entered. Each chromosome was divided according to the ideogram at 400 bands level as described by the ISCN, in such a way that net gains and losses in 1p11-p13 were summarized in 1p1, 1p21-22 was summarized in 1p2, etc. In case of (net) loss in these parts of one of the chromosomes a -1 was entered. If the same parts were lost in two chromosomes a -2 was entered. Similarly, if (net) gain occurred in one of these chromosomal parts a +1 was entered, etc. Only changes as compared to the constitutional karyotype were evaluated. Uncertain breakpoints, structural rearrangements and/or chromosomes were not included in this analysis. If multiple clones were described, the changes were combined and averaged to obtain a tumor representative karyotype. In this way the changes in 86 chromosomal regions per karyotype were recorded. All structural rearrangements were categorized per type of structural chromosome rearrangement (according to the ISCN). Microsoft Excel 5.0a Windows was used to transform the gains and losses of chromosomal material of all non-diploid karyotypes to diploid karyotypes. Before this diploidization, the changes in the chromosomal material of the X-chromosome of men were multiplied by 2 in order to compare male with female karyotypes.

The mean change in chromosomal material per chromosomal region as well as the total and mean number of breakpoints per chromosome arm were calculated. Mean change in chromosomal material in each of the 86 chromosomal regions is expressed as a chromosomal change ratio (CCR) which is defined as the absolute change in a specific chromosomal region as compared to a normal diploid karyotype, e.g. if both chromosomes 1 of all analyzed tumors are lost, the mean change in the specific chromosomal regions 1p1,1p2,1p3, 1q1,1q2,1q3 and 1q4 is: -2.00 CCR in each of the regions. Only full, abnormal karyotypes were used for the analysis of the over- or underrepresentation of chromosomes or chromosomal material. Graphs were constructed using Microsoft Excel 5.0a Windows to visualize the change in

chromosomal material, the amount of breakpoints per chromosome arm and the differences between selected groups, i.e. "GIST" and LMS of soft tissue.

Table 1.
Previously unpublished cytogenetic data of 1 GIST and 3 LMS analyzed at our institute*.

case	Sex / age	diagnosis	karyotype
1	F / 55	GIST - PT, stomach	117~129,XX,der(X;19)t(X;?;19)(p22;?;q13)x3,+der(X;19)(X;?;19)(p22;?;q13),-1,add(1)(q42),del(1)(q41),-2, der(2)t(2;?;7)(p25;?;p13),-3, add(3)(q2?5), add(4)(q1?2)x3, +del(4)(p11 or 12)x2,add(5)(q11),add(5)(q1?1), del(5)(p10), del(5)(p11or12)x2, +del (5)(p10),+i(5)(p10)x4, ?6, add(6)(q12), del(6)(q11)x2,+del(6)(q11),add(7)(p22), +add(7)(p22),+der(7)add(7)(p22)add(7)(q11.2), +i(7)(p10),-8, del(8)(p11.1),?del(8)(q21),+?del(8)(q21)x2,del(9)(q21)x2, del(9) (p13 or p21), +?der(9)del(9)(p21)dic(9;19)(q34;q13), +dup(9)(q34q12),+i(9)(q10),-10,-10,-10,-10,-10,-11,-11,-12, del(12)(p11.2)x2, del(12)(p11.1),+?del(12)(q13),-13,-13,-14, -14,-14,add(14)(p11),-15,-15,-15,-16,-16,add(16)(p13)x3, -17, -17,del(17)(p11),-18,?18), der(18)(?::14q21→14q32::18p11→18qter), add(18)(q23)x2, -19,-19,-19,+20,+20,+add(20)(q13.3)x2,-21,-21,?21,?21,?21, -22,?del(22)(q13)x3, +mar1x2,+mar2x2, +mar3x2, +mar4,+mar5,+mar6 [cp6]
2	M / 59	LMS - PT, left trapezius muscle	41~43,XY,add(1)(p32),der(1)t(?X;1)(p11;p13),-5, ?add(6)(p21),-10,add(13)(p11),+14,dic(14;14)(p13;p13), -22[cp4]/46,XY[9]
3	F / 40	LMS - local recurrence,left knee	47,XX,+2[4]/91~92,add(9)(q21),add(22)(q13)x2[cp5]
4	F / 62	LMS - meta, thoracic wall	46,X,inv(X)(p11q27),t(1;3)(q12;p24)or(q21;p25)[4]/46,XX, inv(1)(p36q23),t(2;10)(q14-21;p11.2)[4]/47,XX,+8[5]/46,XX [10]

GIST: malignant gastrointestinal stromal tumor; LMS: leiomyosarcoma of deep soft tissue; PT: primary tumor; meta: distant metastasis; *Previously published karyotypes are described in the studies of Molenaar *et al.* (1989) and Plaat *et al.*(1999).

Statistics

As an overall test whether the differences in amount of chromosomal material for each chromosomal region and the number of breakpoints between "GIST" and LMS were statistically significant, an analysis of variance was performed where the statistical interaction between the chromosomal position x tumor type was calculated. The calculations were made on the residuals after adjusting for person differences in amount of chromosomal material. Systat 5.1 and 6.0 for Windows were used for calculation. A p-value <0.05 was considered statistically significant. To identify chromosomal regions with significant gains or losses, 95% confidence intervals (CI) were calculated. To avoid the introduction of significant-appearing associations by

chance and to address the problem of multiple comparison, the significance level of 0.05 was adjusted downwards. The number of tests performed is 86. The tests are however statistically dependent, because deletions apply to several chromosomal segments. Therefore a Bonferroni correction factor of 86 is conservative. A mean number of breakpoints in each chromosome arm > 3.1 SD was considered to be significant reflecting a probability threshold of 0.1.

RESULTS

"GIST" : change in chromosomal material per chromosomal region (Fig. 1):

Construction of 95% CI revealed significant loss, expressed as chromosomal change ratio (CCR), of chromosomal material in the chromosomal regions 13q2-q3 (-0.41, -0.48 CCR), 14p1-14q2 (-0.66, -0.53, -0.55, -0.55 CCR), 18p1-q2 (-0.40, -0.40, -0.46 CCR), 22p1 (-0.76 CCR), 22q1(-0.73 CCR). A statistically significant gain of chromosomal material was not observed. Notable gain was observed in 5p1 and of chromosomes 7 and 20.

LMS: change in chromosomal material per chromosomal region (Fig. 2):

After construction of 95% CI, no chromosomal region with significant loss or gain could be identified. Although not statistically significant, there was a notable loss in 5p1, 14p1 and chromosome 22. A notable gain was observed in 8q1-q2 (with very wide CI) and chromosome X.

MPNST: change in chromosomal material per chromosomal region (Fig. 3):

Construction of 95% CI showed significant loss of chromosomal material in the chromosomal regions 9p2 (-0.64 CCR), 11p1 (-0.27 CCR), 11q2 (-0.32 CCR) and 18p1(-0.34). Although not statistically significant, there was also notable loss in 1p3, 9p1, 11q1, 12q2, 13p1, 14p1, 17p1, 18q1-q2, 19p1, 22q1, X and Y. A statistically significant gain of chromosomal material was observed in the 7q1 region (+0.42 CCR), whereas a notable gain was observed in all regions of chromosome 7.

"GIST" compared to LMS and MPNST (Fig. 4):

Statistically significant differences (P<0.001) between the three groups were observed regarding the variance of the gain or loss of chromosomal material. However, it was not possible to confine these differences between the groups to specific chromosomal regions. Therefore, the differences between "GIST" (n=16) and LMS (n = 14) as found in chromosomal regions 5p1, 7p, 14q and 15 were considered meaningful, but not statistically significant. Relative loss of chromosomal material in "GIST" as compared to LMS was found in 8q1-q2, 10q1-q2 and chromosomes 13, 14, 15, 18, 22 and X. Relative loss of chromosomal material in LMS as compared to "GIST" was noticed in 5p1 and 7p1-p1. Shared loss was found in 1p2-p3, 13p1, 14p1 and in chromosome 22. Shared gain was observed in chromosome 20.

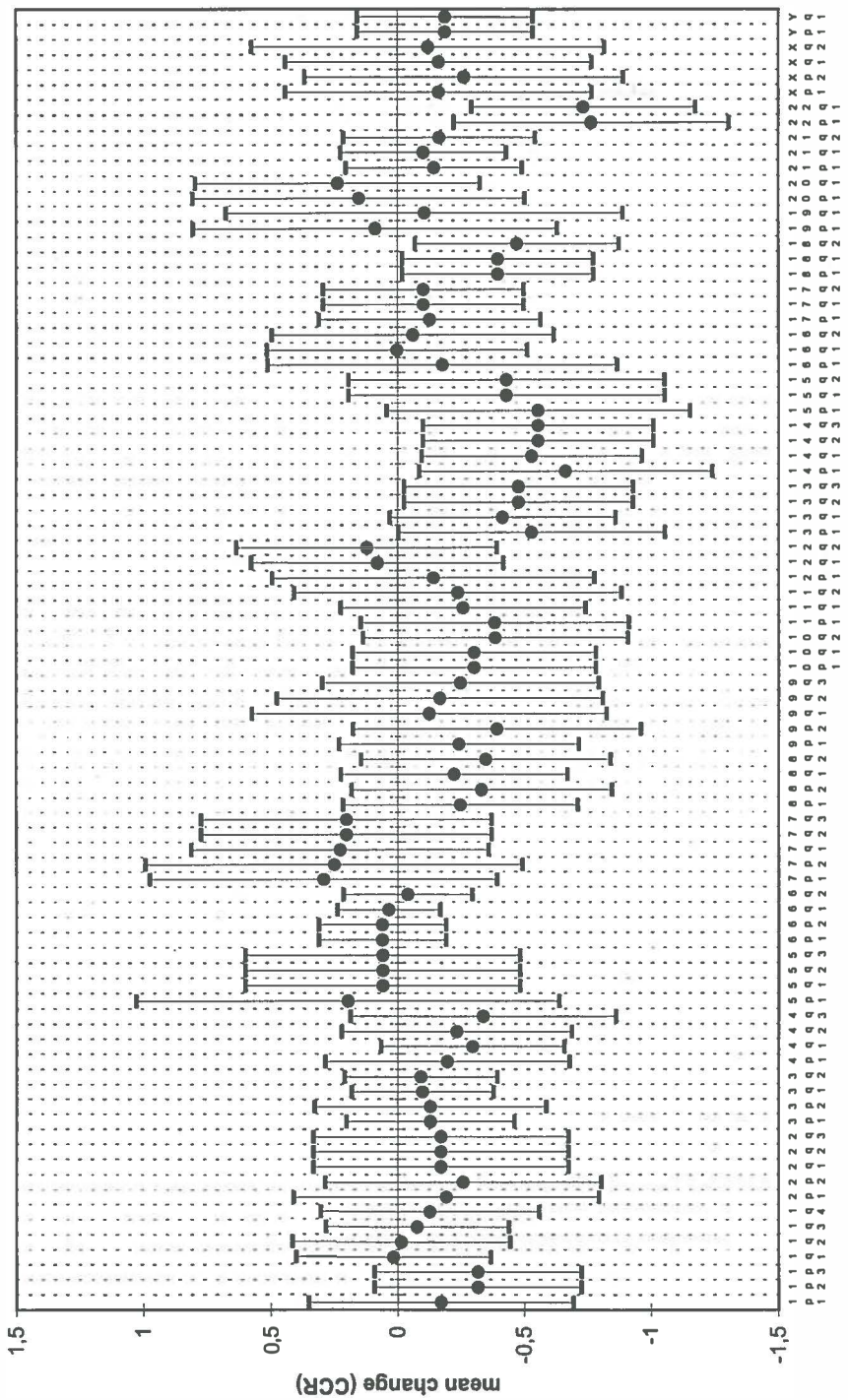


Figure 1
95% confidence intervals of the change in chromosomal material (CCR) in the 86 chromosomal regions after computer assisted cytogenetic analysis of the 16 "GIST".

When "GIST" (n=16) were compared with MPNST (n = 37) notable (but not statistically significant) differences, with a relative loss in "GIST", were found in chromosomal regions 2p2, 8q1-q2, 14q1-q2, 15p1-q2 and 22. A relative gain of chromosomal material in "GIST" was observed in 12q1-q2, 16q1-q2, 17p1, 19 and 20. In both groups there were losses in 1p1-p3, 9p2 (more pronounced in MPNST) and 13p1. Furthermore, in both groups there were losses in 14p1 and 22q1, although these were more pronounced in "GIST". Gain of 7p1-7q3 was observed in both "GIST" and MPNST.

Breakpoints in "GIST" compared to LMS and MPNST (Fig. 5):

Differences in number of breakpoints between the "GIST" and LMS were observed in 1p, 7p, 9q and 14p. Mean numbers of breakpoints per tumor are shown in Table 2. An equal amount of breakpoints in "GIST" and LMS was observed in 1q, 5p and 11q. The breakpoints in these chromosome arms were not clustered at a specific band. Differences in number of breakpoints between "GIST" and MPNST were observed in 1q, 7q, 9p, 9q, 12p, 12q, 14q, 17q and 22q. An equal amount of breakpoints was observed in 1p, 7p and 11q. The breakpoints in these chromosome arms were not clustered at a specific band.

Table 2 - Mean number of breakpoints per tumor in "GIST", LMS and MPNST

chromosome arm	"GIST"	LMS	MPNST
1p	**0.69**	**0.43**	**0.65**
1q	0.31	0.36	**0.65**
5p	0.19	0.21	0.16
7p	**0.44**	0.22	0.38
7q	0.13	0	**0.43**
9p	0.13	0.07	**0.51**
9q	**0.50**	0.14	0.19
11q	0.31	0.36	0.27
12p	0.13	0	0.32
12q	0.06	0.07	0.30
14p	0.06	0.29	0.08
14q	0.13	0.07	0.30
17q	0	0.07	0.32
22q	0.06	0.07	0.30

"GIST": malignant gastrointestinal stromal tumor; LMS: leiomyosarcoma of deep soft tissue; MPNST: malignant peripheral nerve sheath tumor

As shown in Table 3, 1p11-13 was the most involved breakpoint region in both "GIST" (31%) and LMS (21%). In MPNST, 1p11-13 was affected in 19% of the cases, whereas 43% of the MPNST had a breakpoint in 9p11-22. The 9p11-22 region was involved in one "GIST" and one LMS. Breakpoints in 1p32-36 were detected in 1 of 16 (6%) "GIST", 2 of 14 (14%) of the LMS and 8 of 37 (22%) MPNST.

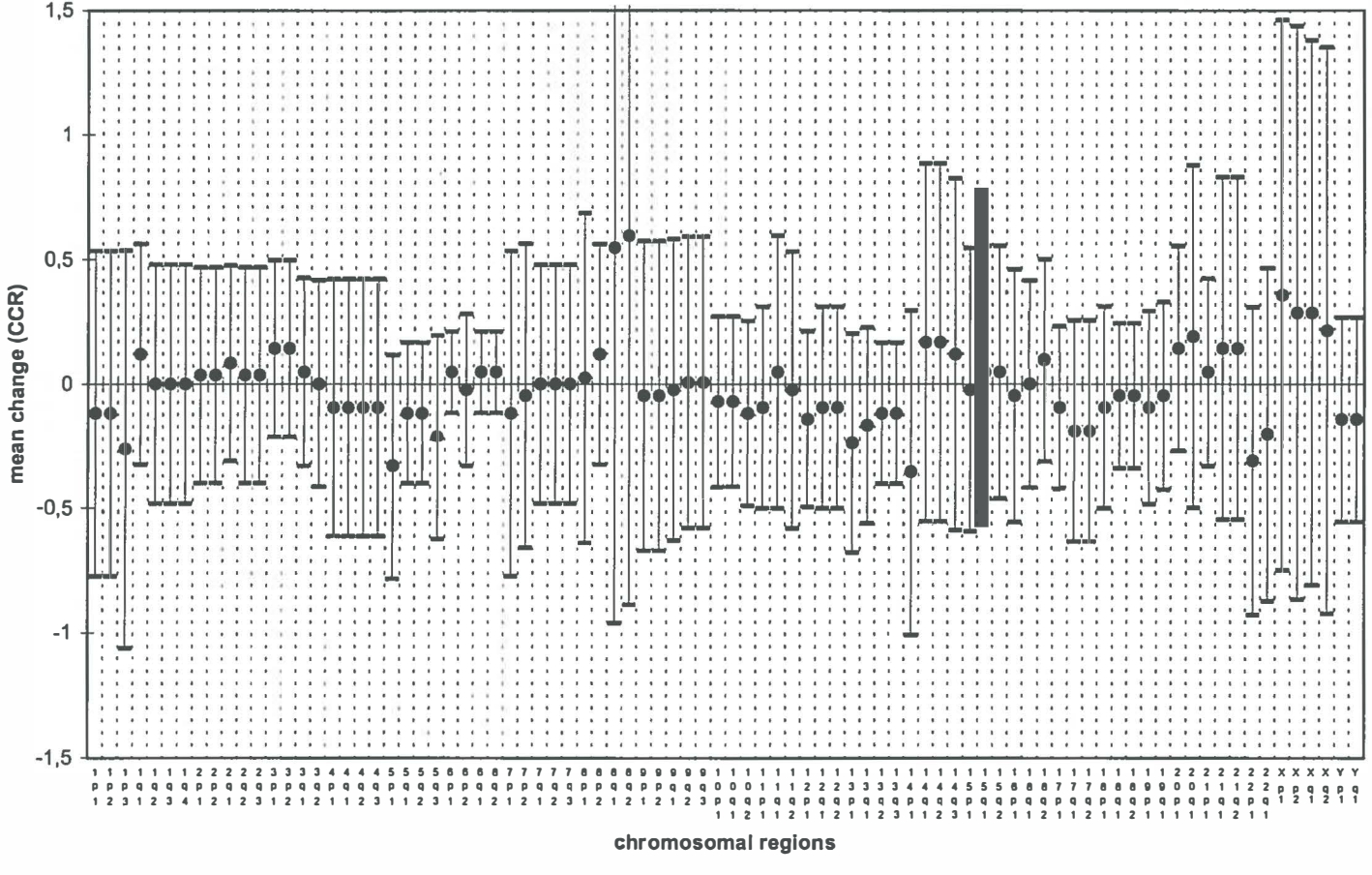


Figure 2
95% confidence intervals of the change in chromosomal material (CCR) in the 86 chromosomal regions in 14 LMS.

Table 3. Percentage of tumors with breakpoints in frequently involved chromosomal regions

Chromosomal region	"GIST" (n=16)	LMS (n=14)	MPNST (n=37)
1p11-13	**31 %**	**21 %**	19 %
1p32-36	6 %	14 %	22 %
1q21-22	6 %	7 %	19 %
7p22	6 %	0 %	16 %
9p11-22	6 %	7 %	**43 %**
11q13-21	6 %	7 %	14 %
17q11	0 %	7 %	14 %
22q11	0 %	0 %	11 %

"GIST": malignant gastrointestinal stromal tumor; LMS: leiomyosarcoma of deep soft tissue; MPNST: malignant peripheral nerve sheath tumor

DISCUSSION

Previous cytogenetic studies have described chromosomal changes in LMS, but did not distinguish between soft tissue, gastrointestinal and uterine tumors 49,53,391-393,395,400-405. The same holds for clinical studies which reported a high level of drug resistance among LMS [87,142,285,286,320]. However, this distinction by site may be clinically relevant. Moreover, evidence now accumulates that most, if not all, mesenchymal tumors of the gastrointestinal tract are derived from interstitial cells of Cajal and are thus histogenetically different from soft tissue LMS which are derived from smooth muscle cells [8,319,327,406,407]. Histopathologically, mesenchymal tumors of the gastrointestinal tract may not only have features of smooth muscle cells, but also neural features [307]. This leads to the concept that gastrointestinal mesenchymal tumors have a common progenitor cell, but nevertheless show divergent phenotypic differentiation. The current study aimed at differentiating between cytogenetic changes 'characteristic' for gastrointestinal mesenchymal tumors, independent of their phenotype, and those that they share with soft tissue LMS and MPNST. Therefore, a computer assisted cytogenetic analysis was done, comparing cytogenetic changes in mesenchymal tumors of the gastrointestinal tract ("GIST"), irrespective of their initial histopathologic classification, with those in LMS and MPNST of soft tissue, combining own data with data retrieved from the literature.

The use of a cytogenetic database in which all karyotypes and other cytogenetic, clinical and histological data are uniformly entered, makes it possible to directly compare and statistically analyze different groups of (already cytogenetically analyzed) tumors, i.e. "GIST", LMS and MPNST without the bias caused by focusing on specific chromosomal abnormalities. Since the objective of this approach was to detect the most common cytogenetic abnormalities in "GIST", LMS and MPNST in a large group of tumors, it was decided to use karyotypes of primary

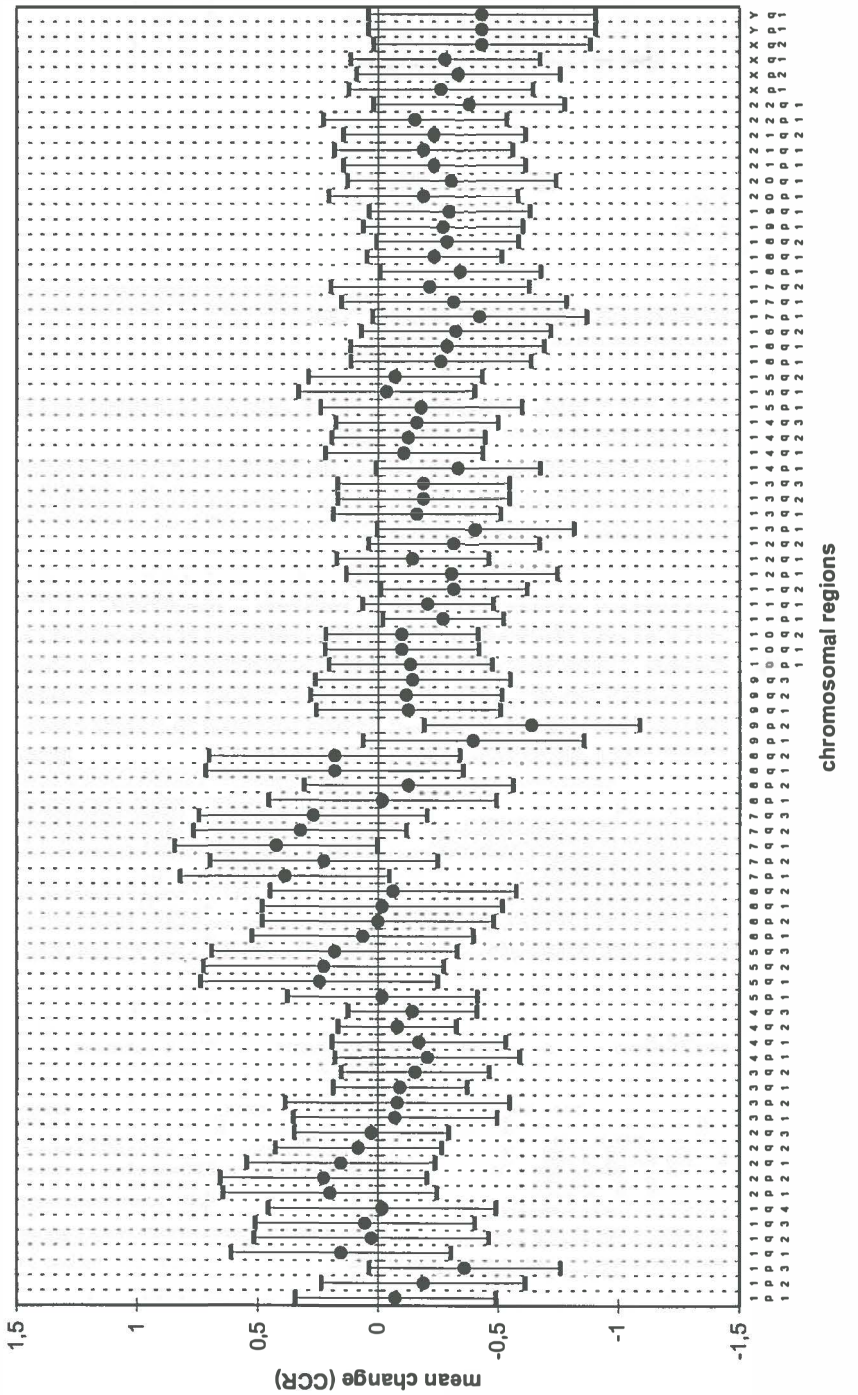


Figure 3
95% confidence intervals of the change in chromosomal material (CCR) in the 86 chromosomal regions in 37 MPNST.

tumors as well as metastatic tumors and tumors of which the tumor status was not clearly described. However, only one entry per patient was analyzed.

This computer assisted cytogenetic meta-analysis evaluates only major effects, since statistical significance could not be reached in the comparison of the three groups due to the relatively small numbers of cases. Nevertheless it has revealed further indications that "GIST" and LMS are different entities. This study did not only restrict the losses in chromosomes 14, 18 and 22 as reported in several individual reports on "GIST", to statistically significant loss in 14p1-q2, 18q2 and 22p1-q1, but also revealed significant loss in 13q2-q3 [317,393-398]. The reported loss of chromosome 15 was confirmed in this study, although it was not statistically significant [395-397]. The observed changes in the 13p, 14p and 15p have to be interpreted with caution, since chromosomes 13, 14 and 15 are acrocentric chromosomes. However, this database supported method detects changes in these regions. This indicates the involvement of these particular chromosomes in a substantial number of structural chromosomal aberrations resulting in a partial loss of these chromosomes, including the p-arm. Loss of the entire chromosomes 14 and 22 might be involved in the oncogenetic processes leading to the development of "GIST". Many genes are located on these chromosomes,
like CDKN3 (14q22), a regulator of the cell cycle, and EWSR1 (22q12), associated with several malignant mesenchymal tumors, i.e. Ewing's sarcomas and related tumors [317,383,408]. Further investigations like the recent studies of El Rifai *et al.* have to narrow the region of interest in "GIST" [317,396,397]. Also the loss of genes on 18q2 and 13q2-q3 might be involved in "GIST" formation. The reported amplifications in 3q2, 5p, 8q2, 17q2, 19q1 could only be confirmed in this study for 5p1 [396,398]. Amplification of 5p1 mapped genes like TERT, which is correlated with telomerase activity in various malignancies, might also be involved in the development of "GIST" [409]. The expression of the c-kit protein, encoded by the *c-kit* proto-oncogene localized on chromosome 4q12, is highly specific for GIST [389]. Although some cytogenetically detected loss in the 4q1 region was observed in "GIST", these changes were not statistically significant. Further studies have to analyze the relation between c-kit protein expression and (cyto)genetic alterations in 4q12. Losses in 14 and 22 were observed in "GIST", LMS and MPNST, but were not statistically significant in LMS and MPNST and therefore have no clear diagnostic value. The shared losses might eventually indicate a common pathway in the development of these tumors.

Differences between "GIST" and LMS, which might indicate a different oncogenesis, were found in the 5p1 region, i.e.gain in "GIST" and loss in LMS. Furthermore, 14q was lost in "GIST", but there was a tendency for gain in LMS and the X chromosome material was amplified in LMS, but not in "GIST". Loss of 14q2, however, was also observed in uterine LMS and might suggest a closer relation between uterine LMS and "GIST" than between soft tissue LMS and "GIST"[405,410-416].

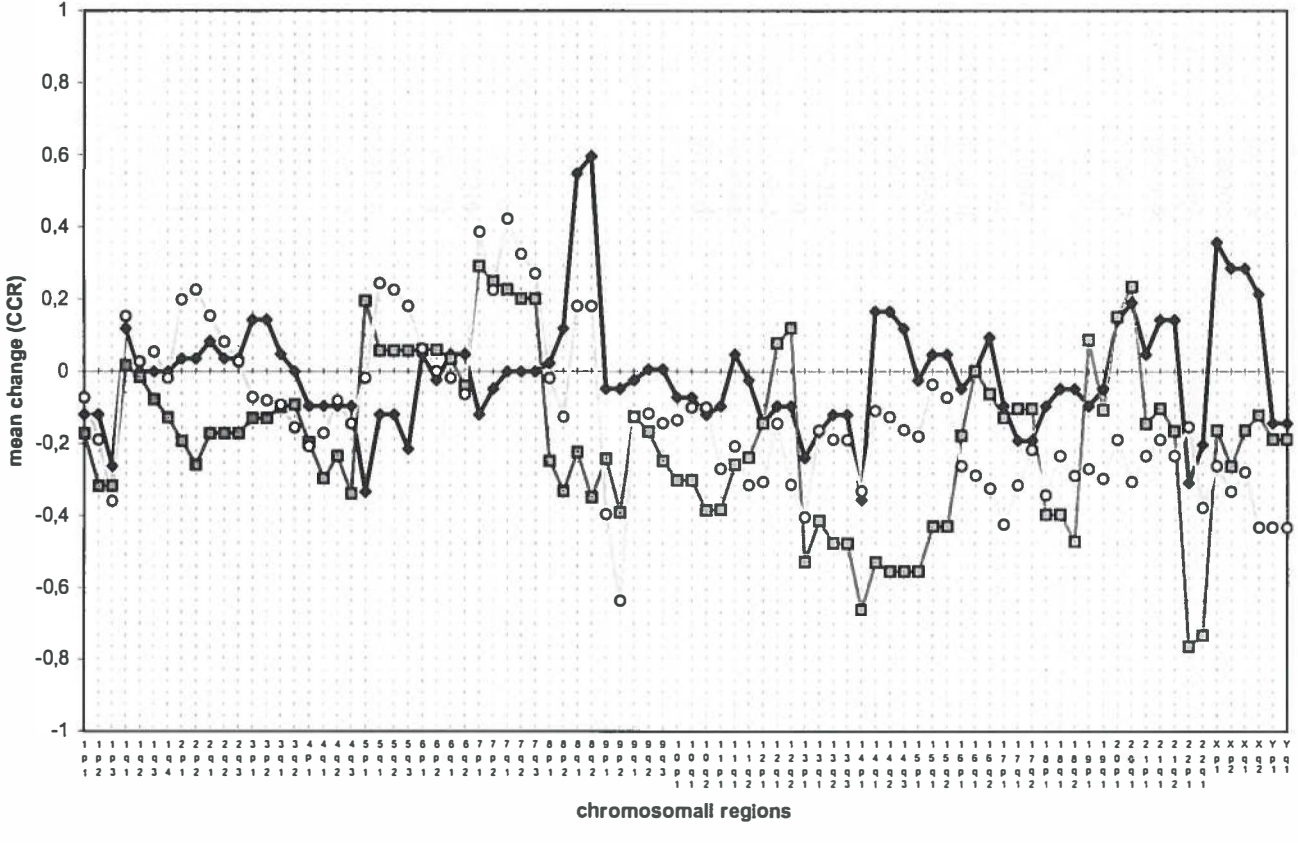


Figure 4
A schematic overview of the mean change in chromosomal material (CCR) in the 86 chromosomal regions: comparison of 16 "GIST" (■, grey line), 14 LMS (◆, black line) and 37 MPNST (●, light grey line).

Although there are no strong indications, it should be noted that chromosomal aberrations in 14q have also been described in colorectal cancer suggesting that 14q anomalies could also be related to the gastrointestinal localization of these tumors [417-419]. Although the gain in 8q1-q2 was mainly due to one case, as is represented by the very wide 95% CI, amplification of 8q was recently reported in a study assessing LMS using comparative genomic hybridization [420]. Since no specific breakpoints and chromosomal gains or losses could be detected in the LMS, the results of earlier studies suggesting involvement of certain chromosomal aberrations specific for LMS could not be confirmed [391,392]. The earlier reported involvement of breakpoints in the 1p12-13, involving the oncogene N-ras on 1p13.2, was also detected in this study as the most involved breakpoint region in both "GIST" and LMS [392,402,421]. However, the finding that this region was only present in 31% of the "GIST" and 21% of the LMS, weakens the theory that this cytogenetically detected breakpoint could be characteristic for the oncogenetic processes of these tumors.

In an earlier study in MPNST, we reported losses in 9p2, 11p1, 11q2, 14p1, 18p1-q2, 22q1, X and Y and gain in the 7q1 region [399]. Differences between MPNST and "GIST", which might have some diagnostic value, were detected in 2p2, 12q1-q2, 15p1-q2, 16q1-q2, 17p1, 19 and 20. In this study similarities in the chromosomal change pattern, indicating a common oncogenetic pathway or neural differentiation of "GIST" and MPNST, were observed in 1p1-p3, 9p2, 13p1, 18p1-q2 (loss) and chromosome 7 (gain). No specific differences or similarities in breakpoints could be observed in "GIST" and MPNST, although the cytogenetically detected breakpoint region 9p11-22 was involved in 43% of the MPNST and in only 6% of the "GIST". Together with the observed loss in 9p2 in MPNST, it can be assumed that genes located on 9p2, like CDKN2A and CDKN2B encoding for p16, might be more affected in MPNST than in "GIST" [381].

In conclusion, the results of this study support the hypothesis that "GIST" is a distinct entity which differs from LMS and MPNST. In "GIST" a significant loss is found in 13q2-q3, 14p1-q2, 18p1-q2 and 22p1-q1, whereas gain of chromosome X seems to be involved predominantly in LMS. MPNST are characterized by gain of chromosome 7 and losses in 9p2, 11p1, 11q2 and 18p1. However, in "GIST", LMS as well as in MPNST losses of chromosomes 22 are observed, indicating a common oncogenetic pathway. Gain of 7q1 and loss of 18q may be more associated with neural differentiation in MPNST and "GIST". Further studies, comparing cytogenetic changes with phenotype in GIST are warranted, as well as molecular studies further probing the significant chromosomal regions.

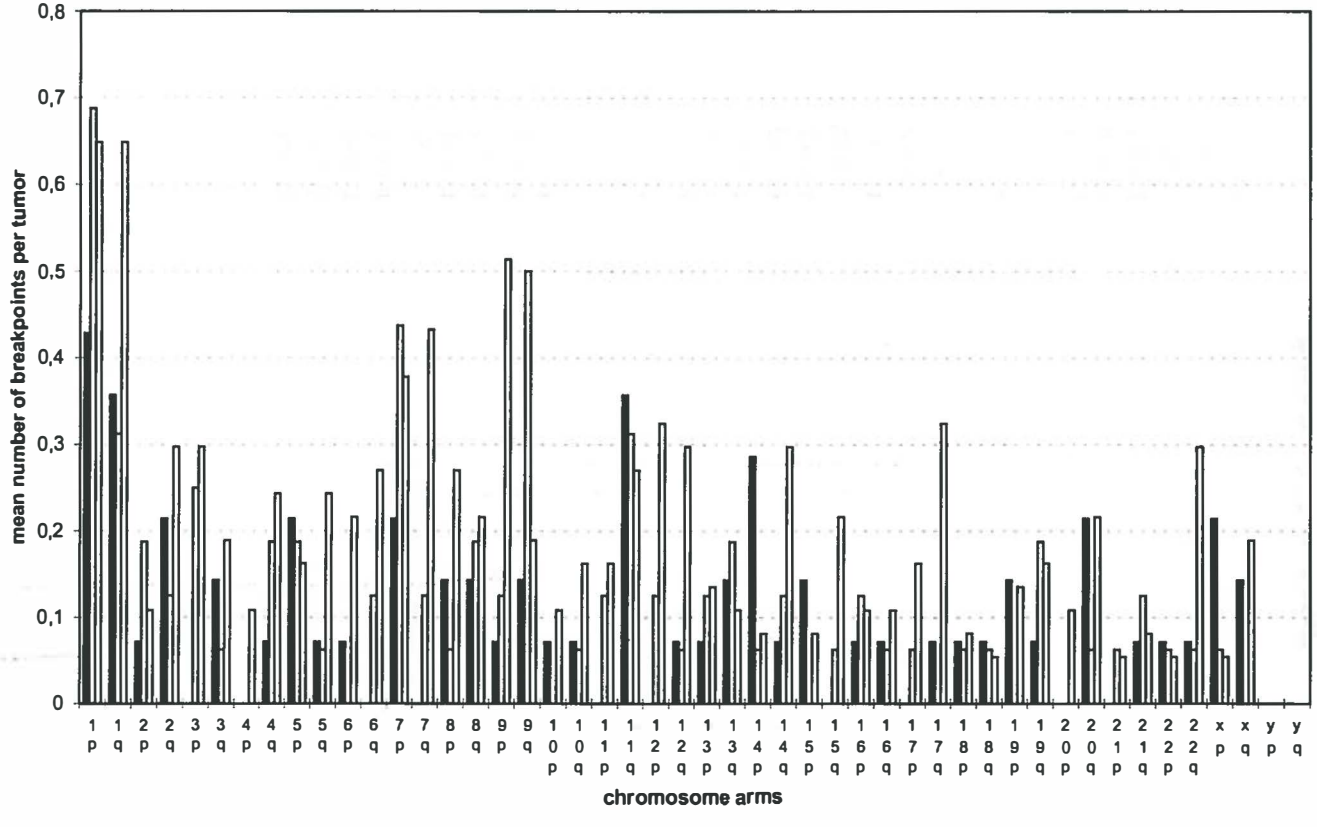
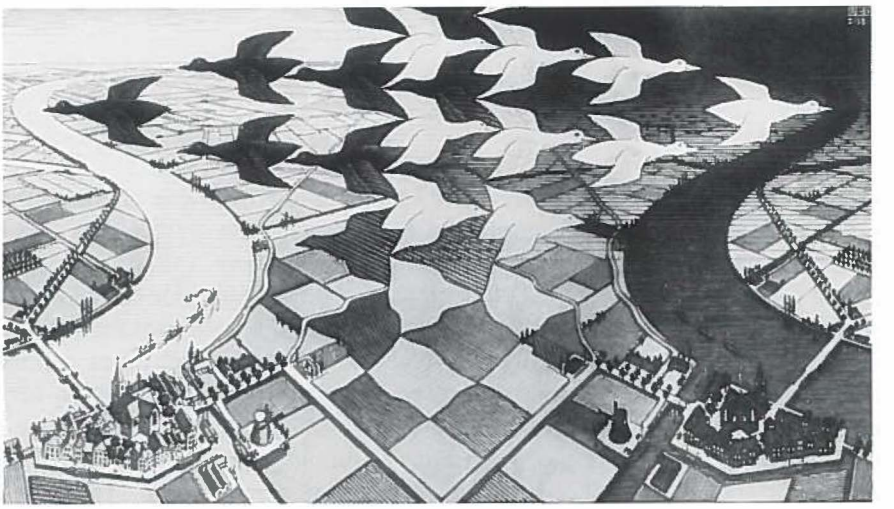


Figure 5
The mean number of breakpoints per tumor in each of the chromosome arms in 16 "GIST" (dark grey bars) compared to 14 LMS (black bars) and 37 MPNST (light grey bars).

Chapter 12

TRANSLOCATION (11;22)(q24;q12) IN A SMALL CELL TUMOR OF THE THIGH IN A 2-YEAR OLD BOY
Immunohistology, cytogenetics, molecular genetics and review of the literature



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Hum Pathol 1999; 30(3):352-5

SUMMARY

A case of a two-year-old boy with a palpable mass in the left thigh is presented. Incisional biopsy was performed and subsequent histopathological examination revealed an infiltrative tumor composed of relatively large cells. The tumor cells were immunoreactive for vimentin and keratin, but not for desmin or smooth muscle actin. Cytogenetic analysis showed a 46,XY,t(11;22)(q24;q12) karyotype. The translocation (11;22)(q24;q12) is said to be characteristic for the family of Ewing's sarcoma and related tumors. As a result of the t(11;22)(q24;q12) the EWS gene on chromosome 22q12 joins the 3' part of FLI-1 gene on chromosome 11q24, which encodes a member of the ets family of transcriptional regulators. Using RT-PCR a corresponding EWS-FLI-1 fusion product was detected. Additional immunohistological staining for p30/p32^{MIC2}, which is suggestive, but not specific for Ewing's sarcoma, appeared to be weakly positive. In the current case a diagnosis of Ewing's sarcoma was considered unlikely, because of the location of the tumor and the immunohistological profile. Nevertheless it was decided to treat the patient according to a Ewing's sarcoma protocol based on the genotype of the tumor. The findings were compared with other extra-osseous pediatric small cell tumors showing the t(11;22)(q24;q12) described in the literature.

INTRODUCTION

The reciprocal translocation (11;22)(q24;q12) is a cytogenetic aberration which is found in 83% of the cases diagnosed as Ewing's sarcoma [422,423] but is also often described in peripheral primitive neuroectodermal tumors (pPNET's) including peripheral neuroepithelioma [424], Askin's tumor [425,426] and esthesioneuroblastoma [427]. These histogenetically related tumors belong to the highly malignant family of small round blue cell tumors which usually involve bone and deeper soft tissue and mainly occur in children and young adults. As a consequence of the translocation between the long arms of chromosomes 11 en 22 the 5' amino- terminal portion of the *EWS* gene on 22q12 fuses with the 3' carboxy terminal region of the *FLI-1* gene, belonging to the *ets* family of transcription factors, on chromosome 11q24. As a result of this fusion the 3' *ets* DNA binding domain of *FLI-1* replaces the 3' RNA-binding domain of *EWS* [408,428,429]. The hybrid *EWS-FLI-1* mRNA transcribed from the derivative chromosome 22 encodes a fusion protein with a strong transcription activator function which can induce malignant transformation in NIH 3T3 cells [428,430]. In a few cases of Ewing's sarcoma and pPNET other translocations have been reported [431].

In this case we present an intermuscular small cell tumor in a 2-year-old boy showing the t(11;22)(q24;q12).

CASE REPORT

In August 1995, a 2-year-old boy was admitted to the Groningen University Hospital because of a painless mass at the dorsal site of the left thigh. The mass had been slowly increasing in size during the past two months. An incisional biopsy was taken and a diagnosis of sarcoma was made; rhabdomyosarcoma could be excluded by immunohistology, but otherwise the tumor was difficult to classify (see below). Imaging studies did not reveal regional or distant metastases. A complete surgical resection was performed. Surgical margins were free of tumor. Cytogenetic analysis of the biopsy revealed a t(11;22)(q24;q12) which was further analyzed by reverse transcription polymerase chain reaction (RT-PCR). Based on the cytogenetic and molecular genetic findings the patient received adjuvant chemotherapy according to a protocol for Ewing's sarcoma. A complete tumor re-evaluation after completion of chemotherapy in September 1996 showed no evidence of residual or recurrent disease. At the time of this report in January 1998, the patient is still in complete remission.

METHODS

Tissue of the incisional biopsy was embedded in paraffin for routine ***histological*** examination. ***Immunohistological*** studies were performed on both frozen, acetone-fixed sections and paraffin embedded sections using the biotin-streptavidin-peroxidase method and diaminobenzidine as chromogen. Primary antibodies (with their source and optimal working dilutions) were directed against vimentin (Boehringer Mannheim, Germany 1:100), keratin AE1/3 (Boehringer Mannheim, 1:400), actin (Enzo Diagnostics, New York, 1:10000), desmin (DAKO 1:100, Glostrup, Denmark), S-100 (DAKO 1:1600), keratin CAM5-2 (Becton/Dickinson 1:4, San Jose, California), HBA-71 anti p30/32^{MIC2} (courtesy of Dr. Hamilton, Vienna 1:250 [432]), neuron specific enolase (Biogenex, San Ramon, 1:100) and neurofilaments (Eurodiagnostics, Apeldoorn, 1:20).

The ***DNA ploidy*** was measured on a tumor cell suspension using a Coulter Epics Elite flowcytometer.

Fresh representative tumor material for ***chromosomal analysis*** was cultured for 6-12 days in RPMI 1640 (Gibco BRL Life Technologies, Gaithersburg, MD, USA), supplemented with 13,5% fetal calf serum, L-glutamin and penicillin/streptomycin. Chromosome preparations were made according to standard cytogenetic techniques. Slides were stained with Giemsa after G banding with pancreatin (0.1%,Sigma).

The karyotypes were described according to the ISCN Guidelines for Cancer Cytogenetics [377].

Molecular study was done using ***Reverse Transciption Polymerase Chain Reaction (RT-PCR).***

Total RNA was extracted from short term cultured tumour cells, using RNazol (Campro Scientific). 2,5 µg RNA was reverse transcribed into cDNA using random

hexamer primers and Ready-to-go You-prime First-strand-beads (Pharmacia). To amplify a *EWS-FLI-1* junction region, PCR was performed using primers 5'EWS and *3'FLI-1/ERG* [433]. As a control for the integrity of the isolated RNA and successful reverse transcription a part of c-ABL, which is ubiquitously expressed, was amplified. The PCR product was analyzed on a (2)% agarose gel and sequenced using an automated sequencing system (ABI 377) to determine the type of hybrid transcript.

RESULTS

Histopathology

Histopathological examination of the incision biopsy revealed a solid proliferation of large cells with vesicular nuclei and prominent nucleoli (Fig.1A). Most cells had amphophilic cytoplasm, but a few cells with strongly eosinophilic cytoplasm were observed. There was a high mitotic rate (25/2 mm^2), but no necrosis. The tumor in the excision specimen measured 2.5x2.5x1.8cm and was composed of several noduli embedded in intermuscular adipose tissue with infiltration in skeletal muscle. The microscopic appearance was identical to that in the biopsy. No tumor cell rosettes were observed. Immunohistology revealed strong diffuse reactivity for keratin AE1/3 (Fig.1B) and for CAM5.2 in virtually all tumor cells as well as for vimentin. Immunoreactive globular intracytoplasmic inclusions were not observed with any of the antibodies. Weak cytoplasmic immunoreactivity for HBA-71, recognizing the $p30/32^{MIC2}$ protein, was seen throughout the tumor and focally for neuron specific enolase and neurofilaments. No desmin or actin immunoreactivity was present.

DNA-ploidy and (cyto)genetics

DNA-ploidy revealed a diploid peak and cytogenetic analysis showed a 46,XY, t(11;22)(q24;q12)[cp9]/46,XY[1] karyotype, with a reciprocal t(11;22)(q24;q12) (Fig.2). In view of the cytogenetic findings molecular studies were performed to confirm the t(11;22) (q24;q12). RT-PCR detected an amplified product of approximately 250 basepairs. After sequencing this product appeared to contain a junction of exon 7 of *EWS* to exon 8 of *FLI-1*.

DISCUSSION

The current report describes an intermuscular tumor located at the dorsal site of the thigh of a 2 year old boy. Histopathologically the tumor was difficult to classify. In view of the morphological aspects, in combination with the widespread dual keratin and vimentin immunoreactivity, a diagnosis of extrarenal malignant rhabdoid

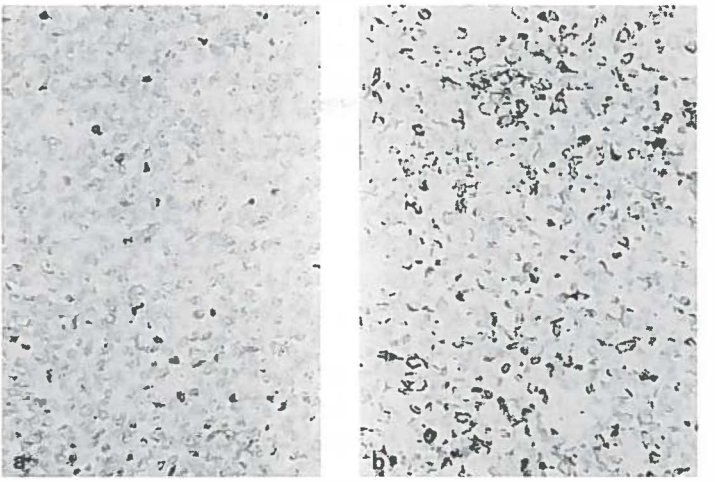


Figure 1
(A) Light microscopy of the tumor showing large cells with vesicular nuclei and prominent nucleoli (hematoxylin-eosin stain; x 400).
(B) Immunohistologicy using the antibody keratin AE1/3 displaying diffuse strong immunoreactivity (ABC immunostaining, counterstained with Mayer s haematoxylin; x 400).

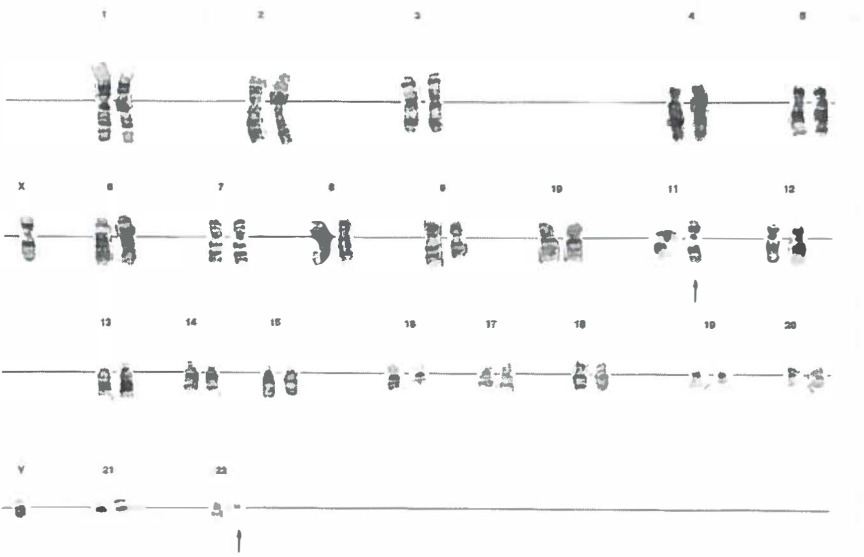


Figure 2
Karyotype of the presented case showing a t(11;22)(q24;q12).

tumor (MRT) was considered. However, immunoreactive globular intracytoplasmic inclusions were not observed with any of the antibodies. In view of the cytogenetic findings additional immunohistochemistry was performed, in an attempt to find support for a diagnosis of Ewing's sarcoma, but these results were inconclusive. Weak,cytoplasmic immunoreactivity for HBA-71 recognizing the p30/32^{mic2} marker is seen in many sarcomas other than Ewing's [434,435] (and personal observations), immunoreactivity for neuron specific enolase is generally known to be aspecific and neurofilaments are present in many sarcomas, including rhabdomyosarcomas and malignant rhabdoid tumors of soft tissue (Molenaar et al.: submitted for publication). Since the histopathological aspects and intermuscular location are also unusual for Ewing's sarcoma this diagnosis was discarded.

Using RT-PCR a *EWS-FLI-1* fusion transcript in which exon 7 of *EWS* is fused to exon 8 of *FLI-1* was detected. This seems to be a rare fusion type in Ewing tumors, only found in 2 out of 147 cases in a European multicenter study [436]. This study suggests a possible advantage in relapse-free survival for patients with localized disease and a shorter (exon 7 of *EWS* fused to exon 6, 7 or 8 of *FLI-1*) chimeric product, like in our case.

Cytogenetic finding of the t(11;22)(q24;q12) and molecular detection of the *EWS-FLI-1* fusion transcript have become important diagnostic markers because they were supposed to be specific for Ewing's sarcoma and related tumors such as peripheral primitive neuroectodermal tumors (pPNET). However, the current case and recent reports in the literature suggest that the (11;22)(q24;q12) may occur in a more heterogeneous group of tumors, as summarized in Table 1. Virtually all tumors occurred in the pediatric or young adult age group and most were difficult to classify. Immunohistologically, a complex phenotype with expression of neural, myogenic or epithelial markers or a combination thereof was often observed (Table 2), leading to diagnoses such as "polyphenotypic tumor" [437]. The "Ewing's sarcoma and pPNET" p30/32^{mic2} marker was seen in a minority of cases. The biological behaviour is difficult to establish in view of the limited number of cases and the relatively short follow-up.

The heterogeneity of pediatric tumors revealing t(11;22) (q24;q12) raises the question whether treatment should be based on histological findings, clinical appearance or cytogenetic and/or molecular analysis. In the case described in this report the molecular and cytogenetic findings led to the decision to give adjuvant chemotherapy as for Ewing's sarcoma. Currently, there is no evidence of recurrent disease in our patient 29 months after the initial diagnosis, which might be due to the "Ewing" therapy.

Table 1. Pathological and cytogenetic characteristics of different pediatric tumors showing the EWS-FLI-1 fusion transcript and their clinical outcome.

Case No./ cell line[a]	Age/ sex	Localization	Diagnosis	cytogenetics t(11;22)	chimeric mRNA *EWS-FLI-1*	follow-up
1	5/F	neck	polyphenotypic small cell tumor	unsuccessful	+	NED, 20 months
2	37mos/M	retroperitoneum	anaplastic polyphenotypic tumor	+	+	NED, 5 months
3	20mos/F	interscapular	mixed embryonal/alveolar RMS	unsucessful	+	DOD, 14 months
4	44mos/F	right parotid area	mixed embryonal/alveolar RMS	+	+	NED, 9 months
5	22mos/F	superficial, the posterior right thigh	malignant sweat gland carcinoma[1]/ superficial variant of ES/pPNET[2]	+	+	NED, 2 years
6	10/F	left-upper chest wall attached to the skin	malignant sweat gland carcinoma[1]/ superficial variant of ES/pPNET[2]	ND	+	NED, 16 years
TC-131	19/M	soft tissue, thigh	alveolar RMS	-	+	UNK
TC-174	9/F	soft tissue, thigh	alveolar RMS	+	+	UNK
TC-206	5/F	soft tissue, arm	malignant ectomesenchymoma[1] /primitive RMS[2]	+	+	DOD, 5 months
TC-253	25/M	retroperitoneum	alveolar RMS	+	+	UNK
TTC-547	13/F	retroperitoneum	alveolar RMS	+	+	UNK
current case	2/M	intermuscular, thigh	malignant rhabdoid tumor	+	+	NED, 23 months

[a] References case 1-4:(Thomer et al., 1996) [74]; case 5 and 6:(Lee et al., 1995)[438]; cell lines TC-131:(Whang Peng et al., 1986; Sorensen et al., 1995) [426, 437]; TC-174,TC-253,TC-547:(Sorensen et al., 1995)[437]; TC-206:(Whang Peng et al., 1992; Sorensen et al., 1995) [437,439].
[1]: preferential diagnosis and [2]: diagnosis later changed in...
ND: not done, NED: no evidence of disease, DOD: death of disease, UNK: unknown.

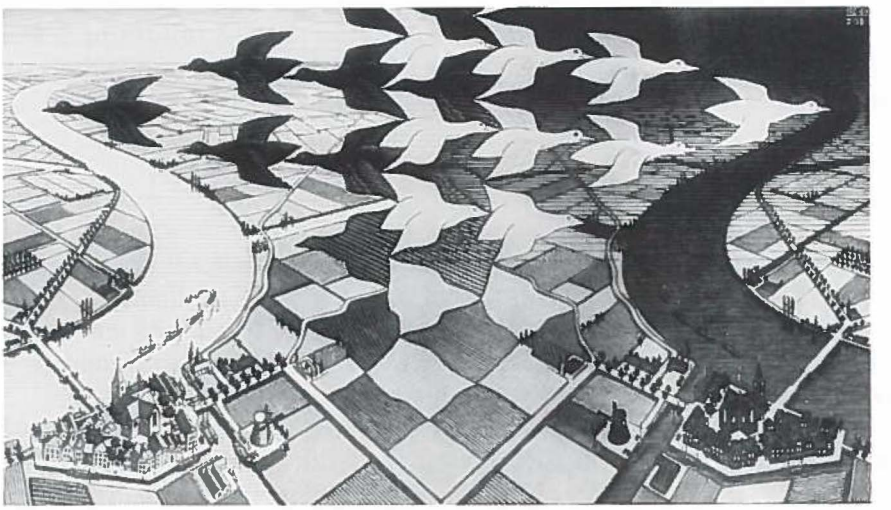
Table 2 Immunoreactivity for different antigens in the various tumors with a t(11;22)(q24;q12).

Case No./ cell line	immunologic profile										
	vimentin	keratin	EMA	CAM5-2	desmin	actin	S100	CD57	MIC2	NF	NSE
1	++	-	+	ND	+	+	+	+	+/-	ND	+
2	++	++	-	ND	++	-	+	+	-	ND	+
3	+++	-	-	ND	+++	+++	-	-	-	ND	-
4	++	-	-	ND	++	+	-	-	-	ND	-
5	++	ND	ND	-	-	+	-	ND	+++	ND	++
6	++	ND	ND	+/-	-	-	-	ND	+++	ND	++
TC-131	ND	ND	ND	ND	++	+	ND	ND	ND	+	ND
TC-174	ND	ND	ND	ND	++	+	ND	ND	ND	+	ND
TC-206	ND	ND	ND	ND	++	++	ND	ND	ND	+	ND
TC-253	ND	ND	ND	ND	+	+	ND	ND	ND	+	ND
TTC-547	ND	ND	ND	ND	++	++	ND	ND	ND	+	ND
current case	+++	+++	-	+++	-	-	-	ND	+	+	+

ND: immunostaining for this antigen not done; +++:strong positive staining; ++ diffuse positive staining;
+: focally positive or weak staining; +/- a rare cell positive and - : negative immunostaining for this antigen

Chapter 13

THE 16p11 BREAKPOINT IN MYXOID LIPOSARCOMAS MIGHT AFFECT THE EXPRESSION OF THE LRP GENE ON 16p11.2 ENCODING THE MULTIDRUG RESISTANCE ASSOCIATED MAJOR VAULT PROTEIN



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SUMMARY

Chromosome breakage could influence the expression of genes. This has been noticed in specific cases of acute myeloid leukemia, where the 16p13 breakpoint affects the expression of the multidrug resistance related protein (MRP). Myxoid liposarcomas (LPS) are characterized by the t(12;16)(q13;p11) which leads to the formation of a FUS-CHOP fusion transcript. This study investigates the relationship between the cytogenetically detected breakpoint 16p11 in myxoid LPS, the presence of the FUS-CHOP fusion transcript in non-myxoid LPS and the expression of the lung cancer resistance major vault protein (LRP) gene on 16p11.2.

Of 16 cases with a diagnosis of a (possible) liposarcoma with an abnormal karyotype, fresh frozen tumor material was available for immunohistological detection of LRP. Cases without a cytogenetically detected t(12;16)(q13;p11), were analyzed for the presence of a FUS-CHOP fusion transcript by RT-PCR.

In all 9 myxoid LPS a t(12;16)(q13;p11) was found and LRP expression was absent or low. In none of the remaining 7 cases a FUS-CHOP fusion transcript was found and four tumors were LRP positive (p=0.02). LRP expression in myxoid LPS (mean: 1.3%) was lower (p=0.07) than in the non-myxoid tumors (mean: 35.7%).

These observations indicate a relation between the t(12;16)(q13;p11), leading to a FUS-CHOP fusion transcript in myxoid LPS, and the low or absent expression of the LRP-gene located on 16p11.2.

INTRODUCTION

Soft tissue sarcomas (STS) are malignant tumors of mesenchymal origin. They comprise 1% of all malignant tumors. About 20% of the STS are liposarcomas (LPS) of which approximately 50% is of the myxoid subtype [8]. Myxoid LPS are histologically characterized by a plexiform network of capillaries and lipoblasts in various stages of differentiation. Cytogenetic studies have revealed a t(12;16)(q13.3;p11.2) in about 95% of the myxoid LPS [8,440]. This translocation which leads to the formation of a FUS-CHOP fusion transcript has also been detected in round cell LPS, which is considered to be a poorly differentiated variant of myxoid LPS [49,441,442]. The remainder LPS consists of well differentiated LPS (WDLPS), pleomorphic LPS (PMLPS) and dedifferentiated LPS (DDLPS). Both WDLPS and myxoid LPS are low grade STS and have a relatively good prognosis as compared to the other LPS, in which distant metastases are frequently observed [8]. Myxoid LPS are characterized by local recurrences, although they can develop distant metastases [8]. Doxorubicin- and carbazine-based chemotherapy has shown effectiveness in LPS [87,443]. Nowadays doxorubicin and/or ifosfamide based chemotherapy regimens are first-line options in the treatment of STS. A serious clinical insensitivity to various chemotherapeutic drugs has been noticed in STS of adult patients [444-446]. Multidrug resistance (MDR) is a phenomenon frequently observed in STS and other malignancies and is associated with the expression of drug efflux pumps like P-

glycoprotein and the multidrug resistance related protein (MRP), which are responsible for the efflux of various natural cytostatic agents among which doxorubicin [92,93,96,97,108,297,322,447-449]. Another protein thought to play a role in MDR is the 110 kD major vault protein LRP (lung cancer resistance protein) [96,97,450]. LRP expression has been shown to be a negative factor concerning prognosis and response to chemotherapy in acute myeloid leukemia (AML) and ovarian cancer [96,97]. In acute myelomonocytic leukemia with abnormal eosinophils (FAB subtype M4eo) an inv(16)(p13q22) has frequently been observed and a relation between the chromosome breakage in chromosome 16p13 and the MRP expression has been found [97,295,448,450,451]. Since the LRP gene is mapped to 16p11.2, there might be a relationship between the breakpoint 16p11 in myxoid LPS, the presence of a FUS-CHOP fusion transcript and the expression of LRP.

MATERIAL AND METHODS

Histology

The criteria for inclusion in the current study were 1) a histological diagnosis of liposarcoma or possible liposarcoma, 2) an abnormal karyotype after cytogenetic analysis and 3) the availability of fresh frozen tumor tissue. In this way, 16 cases were selected and in all cases the histological diagnosis was made on H&E stained paraffin sections with or without additional immunohistological stains. All sarcomas were classified according to Enzinger and Weiss [8] which revealed 9 myxoid LPS, 2 well differentiated LPS, 3 dedifferentiated LPS and two tumors with preferential diagnosis of non-myxoid LPS. Two of the myxoid LPS showed transition to round cell LPS.

Cytogenetics

Fresh representative tumor material for chromosomal analysis was cultured for 6-15 days in RPMI 1640 (Gibco), supplemented with 13.5% fetal calf serum, L-glutamin and penicillin/streptomycin. Cultures were harvested and chromosome preparations were made according to standard cytogenetic techniques. The metaphases were air dried and stained with Giemsa after G banding with either trypsin 1:250 (0.25%, Difco) or pancreatin (0.1%, Sigma). The karyotypes were described according to the ISCN (1995) Guidelines for Cancer Cytogenetics [377]. Cytogenetic results in relation to histology are shown in Table 1.

Molecular genetics: TLS-CHOP fusion transcript

In the 7 cases without a t(12;16)(q13;p11), a TLS/FUS CHOP fusion transcript analysis was performed. From each tumor ten 5µm frozen sections were prepared meticulously obeying Kwok's criteria for avoidance of contaminations [452]. One slice of each case was mounted and HE-stained for histological control. Total RNA was extracted using RNAzol™ B (WAK-Chemie, Bad Homburg, Germany). 0.5 µg to 1 µg of total RNA were reverse transcribed to cDNA using MuLV reverse

transcriptase (Roche Molecular Systems, Branchburg, NJ, USA), random hexamers and RNase inhibitor according to the supplied Roche protocol and 750 µmol/L of each of the four deoxynucleotide triphosphates. One-fourth of the cDNA-mix was used as template for PCR amplification. TLS-CHOP-specific primers, 5'-CAGAGCTCCCAA TCGTCTTACGG-3' (nucleotide position 331-353) and 5'-TTCCAGCTCCCAGCTGGA CAGTG-3' (nucleotide position 992-1014), each 20 pmol/50µl or HPRT housekeeping gene primers, 5'-CGAAGTGTTGGATATAAGCC-3' (nucleotide position 593-612) and 5'-CGATGTCAATAGGACTCCAG-3' (nucleotide position 773-792), each 8 pmol/50µl, and 1.5 U AmpliTaq™ DNA polymerase (Roche Molecular Systems) were used for PCR. Each reaction mix was overlaid by one drop of light mineral oil (Sigma, Deisenhofen, Germany). Amplification cycles for TLS/CHOP were 94°C for 5 min, 70°C for 45 s, 72°C for 1 min (first cycle), 94°C for 1 min, 69.5°C (temperature decrease 0.5°C/cycle), 72°C 1 min (for 25 cycles), 94°C for 1 min, 57.5°C for 45 s, 72°C for 1 min (for 13 cycles), and final extension for 1 cycle at 72°C for 2 min. For HPRT PCR cycles were 94°C for 5 min, 53°C for 1 min, 72°C for 45 s (first cycle), and 94°C for 1 min, 53°C for 1 min, 72°C for 45 s (for 35 cycles) and final extension for 1 cycle at 72°C for 2 min. CDNA synthesis and PCR were performed in a TRIO-Thermoblock™ (Biometra, Göttingen, Germany). PCR products were analyzed on a 2% agarose gel, and visualized by ethidium bromide staining. PCR products indicating a type I (682bp) or type II (406bp) amplicon were digested with 10U MboI (MBI Fermentas,Vilnius, Lithuania) according to the manufacturers protocol and analysed on an agarose gel. RNA preparation, RT and PCR analysis were carried out twice from each tumor.

Detection of LRP

Five micron thick frozen tissue sections were airdried, fixed in acetone for 10 minutes and washed twice with PBS. The slides (except the negative control) were incubated for 1 hr with the primary murine monoclonal antibody LRP-56 (diluted 1:50, kindly provided by Dr. R.J. Scheper, Department of Pathology, Free University Hospital, Amsterdam, The Netherlands). The slides were rinsed three times in PBS with calciumchloride-dihydrate for 5 min and blocked for endogenous peroxidase activity with 0,03% H_2O_2 for 30 min. The sections were rinsed again in PBS with calciumchlorid-dihydrate three times for 5 min, followed by two 30-min incubations with the HRP-labeled rabbit anti-mouse Ig (1:50, DAKO) and HRP-goat anti-rabbit Ig (1:50, DAKO), and subsequent washings in PBS with calciumhydroxide. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole and H_2O_2 for 15 minutes. Nuclear counterstaining was performed with Mayer's Haematoxylin. Normal colon tissue and an LRP-positive soft tissue sarcoma was used as a positive control. Quantification of LRP expression was performed by two observers without knowledge of the histological diagnosis or the cytogenetic results. The LRP expression was analyzed semiquantitatively by estimating the percentage of LRP-positive cells and assessing staining intensity using a scale of 0 -3 (0: no staining, 1: very weak staining, 2: intermediate staining, 3: strong staining). Strong staining intensity was

comparable to the staining intensity of the simultaneously stained positive control tissue. Both staining parameters were also combined into a histopath score which was defined as: the estimated percentage of stained cells (0-100%) x the staining intensity (0-3). According to the literature [96], a tumor was considered LRP-negative if the percentage of positive cells was ≤ 5%.

Statistical analysis

To test differences between groups, the non-parametric Wilcoxon Rank Sum Test and Fisher exact test were used. A p-value of <0.05 was considered statistically significant.

RESULTS

In all 9 myxoid LPS a t(12;16)(q13;p11) was found and LRP expression was absent or low (Table 1). LRP expression was also absent in areas of dedifferentiation towards round cell LPS which were found in two cases. The mean percentage of LRP stained cells was 1.3% (median: 0%, SD: 2.1%). When these values were combined with the amount of staining intensity, the mean histopath score was 2.7 (median: 0 ; SD: 4.2). In none of the seven other tumors a t(12;16)(q13;p11) nor a FUS-CHOP fusion transcript could be detected, but careful cytogenetic analysis revealed a 16p11.1 in one them (case 11). LRP expression in these tumors was found in 4/7 (57%) cases, which was more frequent (p=0.02) than in the myxoid LPS group (0/9). The percentage of LRP stained cells (mean: 35.7%, median: 10%, SD: 40.4%) was higher (p=0.07) than in the myxoid LPS. The histopath score was also higher (p=0.07) than in the myxoid LPS (mean: 39.3, median: 30, SD: 38.3).

DISCUSSION

The sites of chromosome breakage could affect genes located in the affected chromosomal region as has been found in AML. The specific inv(16)(p13q22) in AML is not only of diagnostic importance but also has a prognostic value [451]. The influence of chromosome breakage in the 16p11-16p13 region on LRP and MRP genes has further translated (cyto)genetic findings into a clinical setting [97,295,448,450]. The finding of a characteristic balanced translocation leading to the TLS/FUS-CHOP fusion transcript in myxoid LPS has been of great diagnostic importance. In this study it was questioned whether the breakage in 16p11 and the formation of a FUS-CHOP fusion transcript could alter the transcription of the LRP gene.

Since all myxoid LPS were LRP-negative, the results of this study support the idea that chromosome breakage in 16p11 could affect the 16p11.2 region of the LRP gene and alter the transcription of LRP. In the only non myxoid LPS with a cytogenetically detected 16p11 breakpoint and LRP expression (case 11), the exact breakpoint was 16p11.1, whereas 16p11.2, which is the breakage site involved in the

Table 1a. Cytogenetic and histological data of the myxoid liposarcomas and corresponding LRP expression.

Case	karyotype	diagnosis	TLS/ FUS-CHOP	LRP stained cells (%)	LRP staining intensity	LRP histopath score	LRP status
1	47,XY,t(1;10;**16;12**)(q42;q22;**p11;q13**), del(5)(q21q31),+8[5]	MLPS	ND	0	-	0	-
2	45~47,XY,+8, **t(12;**19;**16)(q13;**q13;**p11**)[cp9]	MLPS	ND	0	-	0	-
3	45~46,XY,der(7)t(7;8)(q36;q22)x2, add(8)(q21) **t(12;16)(q13;p11**)[cp7]	MLPS	ND	5	++	10	-
4	46,XY,t(3;15)(q21;q24), **t(12;16)(q13;p11**)[9]	MLPS	ND	1	++	2	-
5	47,XX,add(3)(p12),der(8)t(3;8)(p14;p12),+der(8)t(3;8)(p14;p12), **t(12;16)(q13;p11**)[6]	MLPS	ND	1	++	2	-
6	44~45,XX,t(5;22)(q31;q13), der(10)t(10;12)(p13;q13), ider(12)**t(12;16)(q13;p11**),-16[cp7]	MLPS	ND	0	-	0	-
7	45~46,XY,**t(12;16)(q13;p11**)[cp7]	MLPS	ND	5	++	10	-
8	45~47,XX,**t(12;16)(q13;p11**), +m1[cp10]	MLPS	ND	0	-	0	-
9	42~47,XY,**t(12;16)(q13;p11**)[cp4]	MLPS	ND	0	-	0	-

MLPS: myxoid liposarcoma; LRP: lung resistance protein; ND: not done; -: negative; +: positive

t(12;16)(q13;p11), is the location of the LRP gene [440]. This was confirmed by the results of the RT-PCR for TLS/FUS-CHOP.

The TLS/FUS-CHOP fusion transcript in myxoid LPS has been the subject of many studies, but a relation with the LRP gene has not been examined before [441,453,454]. This study cannot discriminate between the effects of chromosome breakage in 16p11.2 and the effects of the formation of FUS-CHOP on the expression of LRP. The absence of a FUS-CHOP fusion transcript in cases 10-16 (Table 1) indicates that the 16p11.2 region is not affected and therefore LRP could be expressed in 4 of them.

A preferential histological diagnosis of liposarcoma was made in two cases (cases 12 and 13). In case 12, a patient with an extensive abdominal mass, a tentative diagnosis of RCLPS was made; however, this could not be confirmed by cytogenetics. In case 13 a preferential diagnosis of PMLPS was made, with a differential diagnosis of malignant fibrous histiocytoma. In both cases with an uncertain histological diagnosis, no t(12;16)(q13;p11) nor FUS-CHOP could be detected and LRP expression in these tumors was apparently not hampered by the anomalies in the 16p11.2 region. It remains unclear whether transcription of the LRP gene is present or absent in benign and the various malignant lipomatous tumors in general or is impossible in the myxoid LPS due to the 16p11.2 breakage site characteristic for LPS. In myxoid LPS, the low LRP levels could also be explained by the fact that all myxoid LPS were low grade tumors and some studies reported a correlation between tumor grade and MDR [100,108,455]. However, the latter option seems to be contradicted by the absence of LRP expression in areas with round cell morphology.

Table 1b. Cytogenetic and histological data of the other cases and corresponding LRP expression.

Case	karyotype	diagnosis	TLS/FUS-CHOP	LRP Stained cells (%)	LRP staining intensity	LRP histopath score	LRP status
10	46-48,XX,t(9;15)(p2;q1), tas(15;22)(p13;p13),+2r[cp7] /44-51,XX,+2r,+1-4,mar[cp7]	WDLPS	-	0	-	0	-
11	41-46,XX,add(11)(p11), del(11)(q13q24), *add (16)(p11.1)*, add(20)(q11.2)[cp12]	WDLPS	-	70	+	70	+
12	43-44,X,-Y,der(1;2)(q10;q10),-2,i(4)(q10),-5,+9,-14, dic(19;22)(q13.1;q12), +20,+r[cp7] /46,XY[2]	RCLPS?	-	75	+	75	+
13	20-32(n),X,del(1)(q13), +dic(1;13)(p22;p13),-11, r(12)(p12q24), -13, der(14;21)(q10;q10), -16,-18,-20, -21,+2-6mar[cp6]/38-55,idemx2 [cp5]/80,idemx3[1]/85-86,idemx4 [cp2]/139	PMLPS?	-	90	+	90	+
14	35-62,X,-Y,+X,del(1)(q41),add (2)(q31), del(6)(q16), del(7)(q22),add(8)(q24),der(12) t(9;12)(q12;p13),add(13)(q22),inc[cp 5]/46, XY[10]	DDLPS	-	0	-	0	-
15	72-76,XXY,add(1)(q4), der(1;3)(p10;p10), +dic(3;4)(q21;p15),+2, add(3) (q25),?add(3)(q1),add(5)(q35), +add(5)(q1), add(6)(p22prp23), +7dic(8;?;12)(p1;?;p11.2),+?dic(8;?; 8)(p1;?;p1),+dic(8;?;15)(p11;?;p11), +i(8)(q10),+9,-11,-11, add(11)(p11), der(12;15)(q10;q10), -13,add(15)(p11),+17, +18,-19, -20, -21,i(21) (q10),+22,+r,+1-8mar[cp5]	DDLPS	-	10	+++	30	+
16	clonal aberrations: ±3n,add(5)(p15), add(11)(p1?4) or dic(7;?;11)(p22;?;p1?4), add(11)(p1?4), add(18)(q21), add(22)(q13),mar[5]	DDLPS	-	5	++	10	-

WDLPS: well differentiated LPS; RCLPS: round cell liposarcoma; PMLPS: pleomorphic liposarcoma; DDLPS: dedifferentiated LPS; LRP: lung cancer resistance protein; ND: not done; -: negative; +: positive

Although LRP has shown to be associated with clinical drug resistance in ovarian carcinoma and AML, LRP has also been detected in many normal cell types and is thought to act as an intracellular transporter protein not only carrying MDR cytotoxic substances towards the extracellular environment [95-97,292,294]. Therefore, this observation of a correlation between chromosome breakage and diminished

expression of LRP in solid tumors is not only of oncologic importance, but might be meaningful for the pathophysiological mechanisms in which major vaults are involved.

In summary, in this small group of LPS it was found that expression of the LRP gene on 16p11.2 is absent or very low in myxoid LPS and might be related to the 16p11 breakpoint characteristic for myxoid LPS. These findings might be of pathophysiological, tumor biological and clinical importance. Further studies have to analyze the significance of the MDR protein expression in relation to detected chromosomal breakpoints in LPS. Moreover, studies in larger groups of various benign and malignant lipomatous tumors have to clarify whether LRP expression in lipomatous tumors is low or absent in general.

Acknowledgements

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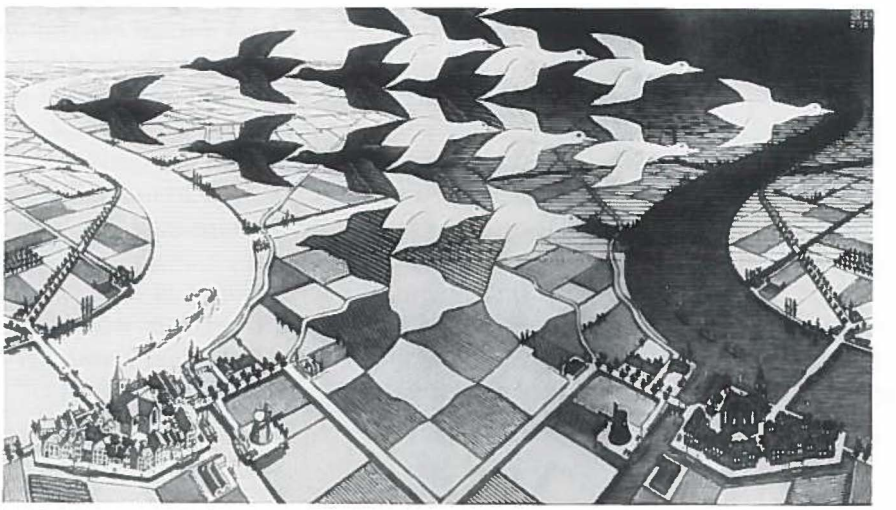
Chapter 14

CHROMOSOMAL ABERRATIONS IN 7q11, 16p11 AND 16p13 IN RELATION TO THE EXPRESSION OF THE MULTIDRUG RESISTANCE ASSOCIATED PROTEINS P-gp, LRP AND MRP_1 IN SOFT TISSUE SARCOMAS



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SUMMARY

Multidrug resistance (MDR) has been associated with the expression of the P-gp (P-glycoprotein), LRP (lung resistance protein) and MRP_1 (multidrug resistance related protein) genes on chromosomes 7q21, 16p11 and 16p13, respectively. Chromosome breakage in 16p13 can influence the expression of MRP_1, as has been shown in specific cases of acute myeloid leukemia. In soft tissue sarcomas (STS), cytogenetic analyses revealed characteristic cytogenetic alterations in several histologic types. This study in 39 STS was performed to evaluate whether cytogenetically detected alterations in 7q21, 16p11 and 16p13 could influence the expression of P-gp, MRP_1 and LRP.

From a cytogenetic database, 24 STS cases were selected with abnormalities, i.e. breakpoints, loss or gain of chromosomal material in 7q21, 16p11 and/or 16p13. Eight of the 16 cases with 16p11 breakpoints were myxoid liposarcomas. As a control group served 15 STS with an abnormal karyotype, but with cytogenetically normal chromosomes 7 and 16. P-gp, MRP_1 and LRP expression was semiquantitatively assessed by immunohistochemical evaluation.

P-gp and MRP_1 expression did not differ between STS with breakpoints in or loss of the 7q21 or 16p13 regions as compared to STS with normal chromosomes 7 and 16. However, in STS with 16p11 breakpoints or loss, the median percentage of LRP expressing cells was 3% and in STS with normal 16p11 regions the median percentage of LRP expressing cells was 40% ($p<0.01$). LRP expression, but not MRP_1 or P-gp expression, correlated with the amount of cytogenetically intact chromosomal material ($p<0.01$). LRP expression in liposarcomas did not differ from that in STS other than liposarcomas, whereas myxoid liposarcomas had lower LRP expression than other liposarcomas ($p<0.01$).

In conclusion, the present study shows that breakpoints in or loss of 16p11 might affect LRP expression in STS. LRP expression correlates with amount of chromosome 16p11 material. In STS, P-gp and MRP_1 expression seems not to be affected by breakpoints in or loss of 7q21 and 16p13, respectively.

INTRODUCTION

Soft tissue sarcomas (STS) are malignant tumors of mesenchymal origin and comprise 1% of all malignant tumors. They tend to spread hematogeneously, predominantly to the lungs, in up to 30% of the patients [8,9]. Metastasized STS are treated with doxorubicin based chemotherapy and response rates are in general 20-30% [86,87,281-284]. Poor response to various functionally and structurally distinct natural cytostatic agents has been called multidrug resistance (MDR) [288,350]. MDR has been associated with the overexpression of proteins like P-glycoprotein (P-gp), the product of the MDR1 gene located on 7q21, [288,325,326,350] and the multidrug resistance related protein-1 (MRP_1) gene, located on 16p13 [287,289-291,351]. Some studies reported a prognostic value of P-gp and MRP_1 in STS [100-103,108,297,324]. In addition, a possible role

in clinical drug resistance of another gene on 16p11.2 encoding the major vault protein or lung resistance protein (LRP) has been reported [96,97,289,292-295]. In acute myeloid leukemia (AML) with abnormal eosinophils (FAB subtype M4eo) with an inv(16)(p13q22) it was found that breakage in chromosome 16p13 affects MRP_1 expression, which was shown to be of prognostic significance [450,451]. Previous studies on cytogenetic abnormalities in STS have focussed on the significance for histological classification and tumor progression [42,49,53,54,456-458]. In myxoid liposarcomas (LPS) a t(12;16)(q13.3;p11.2) is found in about 95% of the cases, which involves the chromosomal region 16p11.2 on which the LRP gene has been located [8,440]. Since clinical drug resistance in STS has been frequently observed, it was decided to evaluate in 39 STS the relation between chromosome breakage and/or loss or gain of 7q21, 16p11 and 16p13 with the expression of P-gp, MRP_1 and LRP, respectively.

MATERIALS AND METHODS

Patients and study design

The criteria for inclusion in the current study were 1) a histological diagnosis of a STS, 2) an abnormal karyotype with abnormalities of chromosomal regions 7q21, 16p11 and/or 16p13 and 3) the availability of paraffin embedded tumor tissue. In this way a study group was selected of a) 20 cases with breakpoints in or loss of the chromosomal regions 7q21, 16p11 and/or 16p13 and b) 5 cases with gain of 7q21, 16p11 and/or 16p13. As a control group served 14 STS with an abnormal karyotype, but with cytogenetically normal chromosomes 7 and 16. For the assessment of the relation between chromosomal damage and the P-gp expression in STS, the cases with breakpoints in or loss of the specific region i.e. 7q21 were compared with all STS with cytogenetically normal 7q21 regions, thus excluding the cases with gain of chromosomal material. The same holds for the 16p11 and the 16p13 region for the analysis of LRP and MRP_1 expression. Thus, breakpoints were considered as loss of chromosomal material in the specific chromosomal region. The relation between gain of 7q21, 16p11 and/or 16p13 and expression of the P-gp, MRP_1 and LRP was evaluated in a correlation analysis.

In all cases the histological diagnosis was made on haematoxylin-eosin stained paraffin sections with or without additional immunohistological stains. All STS were classified according to Enzinger and Weiss [8] (Table 1). The primary STS were graded according to the grading system of Coindre et al. [29]. Tumor material of three primary tumors was obtained after hyperthermic isolated limb perfusion with tumor necrosis factor alfa and melphalan [80,84]. The karyotypes of these three STS showed no alteration in chromosomes 7 or 16.

Cytogenetics

For the cytogenetic analyses, fresh representative tumor material for chromosomal analysis was cultured for 6-15 days in RPMI 1640 (Gibco, Paisley, Scotland), supplemented with 13.5% fetal calf serum, L-glutamin and

penicillin/streptomycin. Cultures were harvested and chromosome preparations were made according to standard cytogenetic techniques. The metaphases were air dried and stained with Giemsa after G banding with either trypsin 1:250 (0.25%, Difco, Detroit IL, USA) or pancreatin (0.1%, Sigma, St. Louis, USA). The karyotypes were described according to the ISCN (1995) Guidelines for Cancer Cytogenetics [377].

MDR expression

For detection of P-gp, the monoclonal antibody C494 (Signet Laboratories, Dedham MA, USA) in a concentration of 120 μg/ml in phosphate-buffered saline (PBS) (Merck, Darmstadt, Germany) plus 1% bovine serum albumin (BSA) (Serva Electrophoresis GmbH, Hamburg, Germany) was used. For detection of MRP_1 the monoclonal antibody MRPr1 (provided by Dr. R.J.Scheper, Dept. of Pathology, Free University Hospital, Amsterdam) in a concentration of 20 μg/ml in 1% BSA/PBS was utilized. For detection of *LRP* the monoclonal antibody LRP (Transduction Laboratories, Los Angeles CA, USA) in a concentration of 250 μg/ml in 1% BSA/PBS was used. As controls for immunohistochemal detection served cytospins of the well documented cell lines A2780 and GLC4 and their corresponding multi-drug resistant sublines i.e. overexpressing P-gp (A2780 AD), MRP_1 (GLC4/ADR) and LRP (GLC4/ADR) [298,299], as well as paraffin embedded formalin fixed liver (P-gp), lung (MRP_1) and colon (LRP) tissue.

Paraffin sections (3 μm) were placed on positively charged glass slides and were dried. Immunohistochemistry was performed according to a method modified from Shi et al. [177,178] . Briefly, after heating on a hotplate, the slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After each of the subsequent steps, three 5 minutes washes in PBS were carried out. After three times heating in an autoclave for 5 minutes at 115° C in a 20 mM blocking reagens (Boehringer Mannheim, Mannheim, Germany) with pH=6.0, endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in PBS during 30 minutes. The slides were incubated with the specific antibody in 1% BSA/PBS (pH=7.4) for 1 hour at room temperature in a humidified chamber. The primary antibody was detected with a rabbit antimouse (DAKO, Glostrup, Denmark) (C494 and LRP) or rabbit antirat (DAKO, Glostrup, Denmark) (MRPr1) peroxidase labeled secondary antibody diluted in 1:50 + 1% human serum (type AB) followed by incubation with goat antirabbit conjugated peroxidase diluted in 1:50 +1% human serum (type AB). 3.3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA) with imidazole (Merck KgaA, Darmstadt, Germany) in PBS was used as the chromagen according to the manufacturer's instructions. After counterstaining with Mayer's haematoxylin, the slides were dehydrated through graded alcohols and mounted with coverslips.

The expression of P-gp, MRP_1 and LRP was assessed independently by two observers (B.P, H.H.) without knowledge of the histological diagnosis or cytogenetic data. The P-gp, MRP_1, LRP proteins were studied in adjacent slides of the most viable parts of the tumor. Amount of P-gp, MRP_1 and LRP expression was

Table 1. Patient characteristics, histology and cytogenetic data of the 39 STS.

case	sex	age	diagnosis	tumor grade	case/control	7q21 breakpoint	amount of 7q21	16p11 breakpoint	amount of 16p11	16p13 breakpoint	amount of 16p13	P-gp	LRP	MRP_1
1	f	24	Rhabdomyosarcoma	II	7q21		-2/3		-1 1/3		-1 1/3	50	80	70
2	f	51	fibrosarcoma	III	7q21	●	+1					30	5	70
3	f	34	liposarcoma		7q21		-1					20	80	70
4	m	62	sarcoma NOS	III	7q21,16p13	●				●	-2	0	20	1
5	m	52	Myxoid liposarcoma		7q21,16p13, gain 16p11		-1		+1/2	●	-1	90	60	5
6	f	52	liposarcoma	II	gain 7q21		+2					5	80	30
7	m	67	liposarcoma	III	gain 7q21		+2/3					40	0	0
8	m	54	MPNST	II	gain 7q21		+2/3					30	5	20
9	f	69	sarcoma NOS	III	gain 7q21, 16p11, 16p13		+2/3		+2/3		+2/3	0	40	0
10	f	25	MPNST	II	gain 7q21, 16p11, 16p13		+1 1/3		+2/3		+2/3	60	10	5
11	f	51	Myxoid liposarcoma	I	16p11			●	-1			90	0	0
12	m	82	Myxoid liposarcoma		16p11			●	-1			0	5	5
13	m	71	Myxoid liposarcoma		16p11			●	-1			5	0	0
14	m	35	Myxoid liposarcoma	I	16p11			●	-1			60	0	0
15	m	26	Myxoid liposarcoma	I	16p11			●	-1			50	0	70
16	m	49	Myxoid liposarcoma	II	16p11			●	-1			0	5	0
17	m	37	Myxoid liposarcoma	I	16p11			●	-1			80	0	0
18	f	51	Myxoid liposarcoma *	III	16p11			●	-1			20	0	20
19	f	37	Myxoid liposarcoma	I	16p11			●	-1		-1	80	5	0
20	f	47	Liposarcoma	I	16p11, 16p13			●	-1		-1	80	40	50
21	f	32	MPNST	II	16p11, 16p13		+2/3		-2/3		-2/3	0	10	0
22	m	42	MPNST	III	16p11, 16p13				-2		-2	90	20	70
23	m	16	Synovial sarcoma	III	16p11, 16p13		+1		-1		-1	80	0	5
24	f	1	Rhabdomyosarcoma	II	16p11, 16p13				-1		-1	5	30	1
25	m	68	liposarcoma	III	16p13					●	-1	40	80	90
26	f	70	liposarcoma	I	control							0	0	0
27	f	43	liposarcoma	II	control							70	50	5
28	m	48	liposarcoma	II	control							60	90	5
29	m	69	liposarcoma	II	control							60	40	5
30	f	84	sarcoma NOS	II	control							60	90	10
31	f	59	sarcoma NOS	II	control							0	30	10
32	f	26	Synovial sarcoma	III	control							10	90	0
33	m	43	Synovial sarcoma		control							40	0	0
34	f	28	Mal. Haemangiopericytoma	II	control							40	40	40
35	m	50	Mal. Haemangiopericytoma		control							0	0	0
36	m	22	Epithelioid sarcoma		control							20	70	70
37	f	27	angiosarcoma	I	control							60	90	40
38	m	60	Leiomyosarcoma	II	control							80	90	40
39	m	89	Mal. Fibrous histiocytoma	III	control							40	50	10

P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein,m: male, f: female, abnorm.: abnormalities, Mal.: malignant, *myxoid liposarcoma with round cell component NOS: not otherwise specified, MPNST: malignant peripheral nerve sheath tumor; ●: breakpoint

semiquantitatively assessed by estimating the percentage of positive stained tumor cells and assessing staining intensity using a scale of 0 -3 (0: no staining, 1: very weak staining, 2: intermediate staining, 3: strong staining). Strong staining intensity was comparable to the staining intensity of the simultaneously stained positive control tissue. Both staining parameters were also combined into a so called histopath-score which is defined as: the estimated percentage of stained cells (0-100%) x the staining intensity (0-3) [300]. According to the literature, tumor samples were considered negative for expression of each of the proteins if ≤ 5% of the cells were positive [96,103].

Statistics

A Fisher exact test was used to analyze the distribution of the examined cases within the groups. A Mann Whitney Test was carried out to analyze the differences in MDR parameters between the tumors with and without abnormal chromosomal regions i.e. 7q21, 16p11, 16p13. To avoid the problem of multi-comparison in the analyses of P-gp, MRP_1 and LRP expression, a Bonferroni correction factor of 3 was introduced. To quantitate the degree of correlation between MDR protein expression and the amount of chromosomal gain or loss, the Spearman's rank test was used. A two-tailed p-value of < 0.05 was considered to be significant. Statistical software SPSS 9.0 for Windows (SPSS Inc., Chicago IL, USA) was used for statistical analysis.

RESULTS

One out of 11 (9%) P-gp negative STS and 4/28 (14%) P-gp positive STS had breakpoints or loss of 7q21 (N.S.). As shown in Table 2 and Figure 1, P-gp expression in the 5 STS with breakpoints in or loss of 7q21 was not different from the P-gp expression in the 27 STS with normal chromosomes 7, as assessed by both the percentage of positive cells and the histopath score (Figure 1a). When the P-gp expression was related to chromosomal gain or loss in 7q21, no correlation was observed with either percentage of positive cells (Figure 1b) or histopath score.

Ten out of 16 (63%) LRP negative STS and 5/23 (22%) LRP positive STS had breakpoints or loss of 16p11 ($p=0.02$). As has been depicted in Figure 2, the expression of LRP, as assessed by the percentage of positive cells and histopath score, in the 15 STS with a breakpoint in or loss of 16p11 was significantly lower than in the 21 STS with normal chromosomes 16 ($p=0.03$)(Table 2). In the 39 STS, a significant correlation was observed between the amount of cytogenetically intact chromosomal material in 16p11 and the LRP expression (Figure 2c).

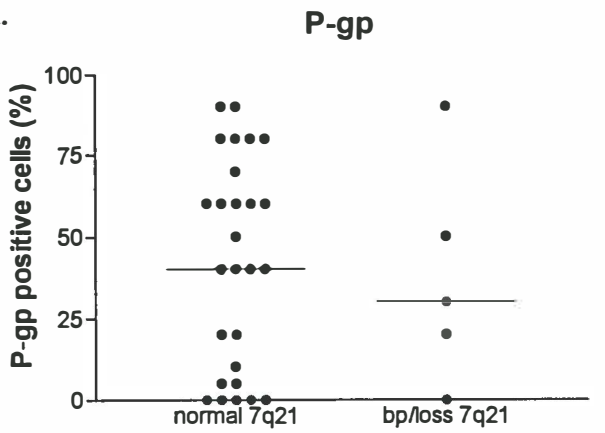
Six out of 22 (27%) MRP_1 negative STS and 4/17 (24%) MRP_1 positive STS had breakpoints or loss of 16p13 (N.S.). No differences in MRP_1 expression, as assessed by either the percentage of positive cells or histopath-score, were observed between the 10 STS with breakpoints in or loss of 16p13 and the 27 STS with normal

Table 2.
The effect of aberrations in 7q21, 16p11 and 16p13 on the expression of P-gp, LRP and MRP_1

	P-gp		LRP		MRP_1	
	no aberrations in 7q21	aberrations in 7q21	no aberrations in 16p11	aberrations in 16p11	no aberrations in 16p13	Aberrations in 16p13
tumors (n):	27	5	21	15	27	10
positive cells:						
mean (%):	42	38	48*	13*	19	29
median (%):	40	30	50*	5*	5	5
SD (%):	31.9	34.2	36.4	22.3	25.2	36.4
range (%):	0-90	0-90	0-90	0-80	0-70	0-90
[a]histopath score:						
mean:	79	52	113*	20*	30	48
median:	60	40	120*	10*	10	5
SD:	77.3	42.1	96.0	40.7	48.4	83.5
range:	0-270	0-100	0-270	0-160	0-210	0-270

P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein;
[a]histopath score: % positive cells x staining intensity (0-3), *p=0.03

A.



B.

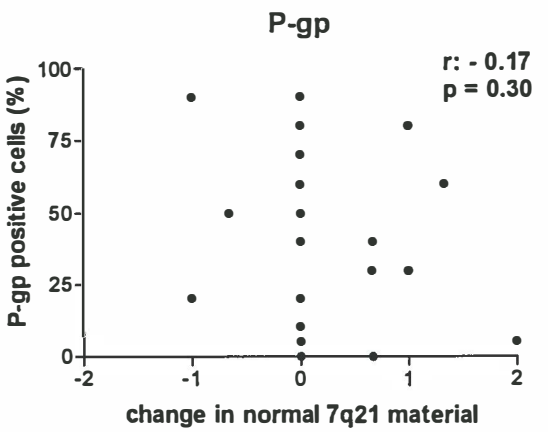


Figure 1: P-gp expression in relation to (a) chromosomal aberrations in 7q21 and (b) the amount of intact chromosomal material of 7q21.

chromosomes 16 (Table 2; Figure 3a). MRP_1 expression did not correlate with the amount of intact chromosomal material in 16p13 (Figure 3b).

Ten (26%) of the 39 studied STS were myxoid LPS and in nine of them a t(12;16)(q13;p11) was found. No differences in P-gp and MRP_1 expression were observed between the myxoid LPS group and the other LPS or the other STS, but LRP expression in the myxoid LPS was lower (p=0.03). P-gp, MRP_1 or LRP expression in the non-myxoid LPS was comparable with the expression in the STS other than liposarcomas (Table 3). P-gp expression, as determined by the amount of positive cells and the histopath score, did not correlate with LRP or MRP_1 expression. However, MRP_1 expression did correlate with LRP expression with respect to both the percentage positive cells (r: 0.49; p=0.005) and histopath score (r: 0.52; p=0.002) and the correlation was still present when the myxoid LPS were omitted (r: 0.46; p=0.005). P-gp, MRP_1 and LRP expression did not correlate with tumor grade.

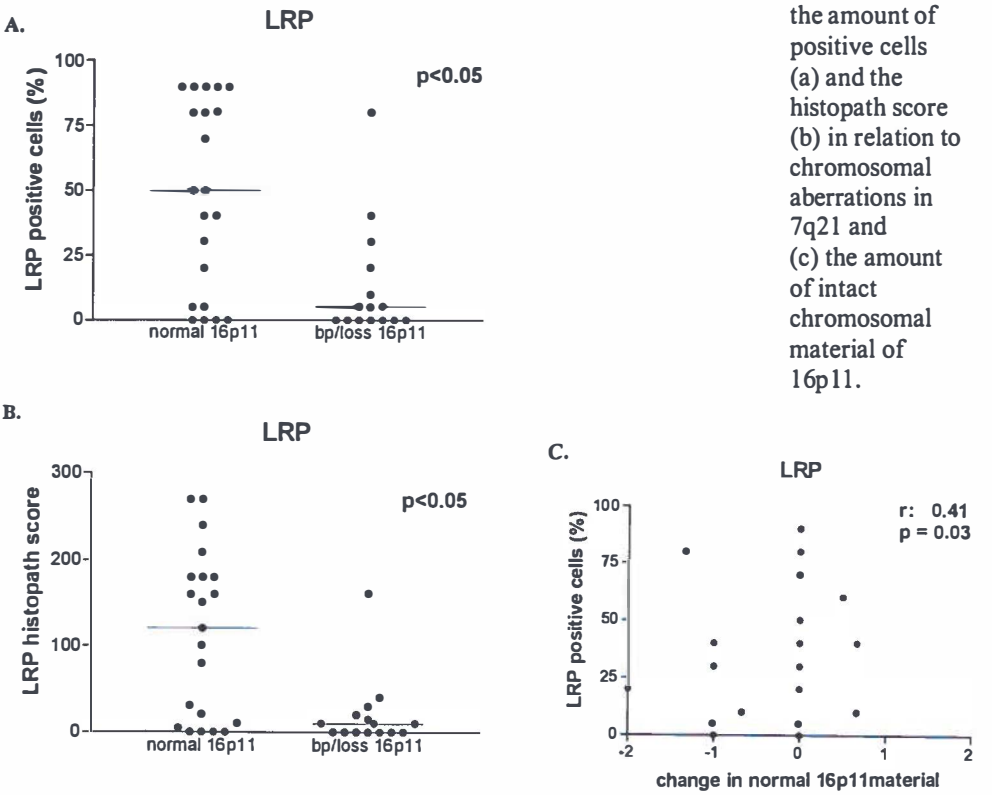


Figure 2: LRP expression as assessed by the amount of positive cells (a) and the histopath score (b) in relation to chromosomal aberrations in 7q21 and (c) the amount of intact chromosomal material of 16p11.

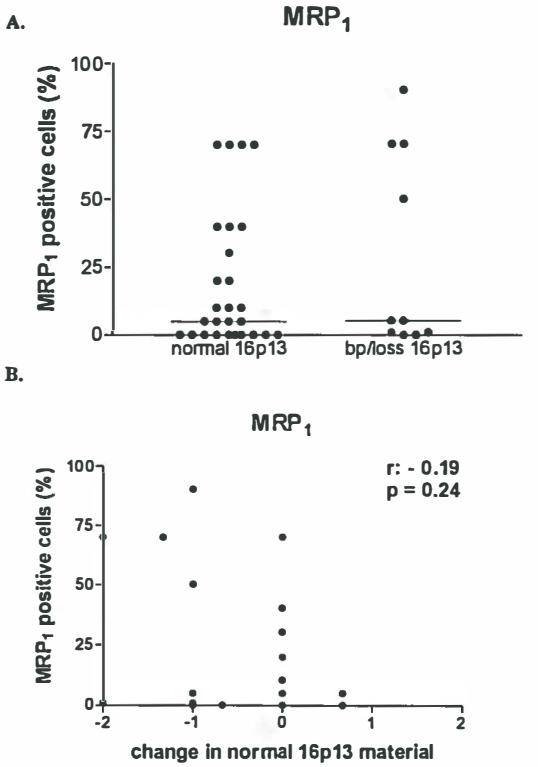


Figure 3: MRP_1 expression in relation to (a) chromosomal aberrations in 7q21 and (b) the amount of intact chromosomal material of 16p13.

Table 3.
The expression of P-gp, LRP and MRP_1 in myxoid LPS, other LPS and non-lipomatous STS.

	P-gp			LRP			MRP_1		
	myxoid LPS	other LPS	other STS	myxoid LPS	other LPS	Other STS	Myxoid LPS	other LPS	other STS
tumors (n):	10	9	20	10	9	20	10	9	20
positive cells:									
mean (%):	48	42	35	8*#	51*	39#	10	28	23
median (%):	55	40	35	0*#	50*	30#	0	5	10
SD (%):	38.0	28.5	30.1	18.6	34.4	34.5	22.0	34.0	27.6
range (%):	0-90	0-80	0-90	0-60	0-90	0-90	0-70	0-90	0-70
[a]histopath score:									
mean:	88	64	57	16*#	107*	87#	20	52	32
median:	75	60	35	0*#	100*	30#	0	5	15
SD:	83.2	51.7	69.8	37.2	84.3	95.1	44.1	86.6	48.7
range:	0-240	0-160	0-270	0-120	0-240	0-270	0-140	0-270	0-210

P-gp: P-glycoprotein, MRP_1: multidrug resistance protein, LRP: lung resistance protein; [a]histopath score: % positive cells x staining intensity (0-3), * difference between myxoid LPS and other LPS: p=0.04; # difference between myxoid LPS and other STS: p=0.03

DISCUSSION

Cytogenetic analysis led to the detection of tumor specific chromosomal alterations which are of diagnostic value and has been helpful in clarifying oncogenesis of various malignant solid tumors [61,358-360]. The sites of chromosome breakage could affect genes located in the affected chromosomal regions. In AML, the inv(16)(p13q22) is not only of diagnostic importance, but also has a prognostic value [451]. Chromosome breakage in the 16p11-16p13 region could affect the expression of the genes for LRP and MRP_1 genes which might be of clinical importance [97,295,448,450]. In a recent study of Van der Kolk *et al.* (personal communication) the activity of MRP_1 in AML patient samples was shown to correlate with the presence of the number of fluorescence in situ hybridization (FISH) signals, suggesting that there is a relation between number of altered chromosomes and the functional activity of MRP_1.
The current study, using conventional cytogenetics, examined whether chromosome alterations in 7q21, 16p11 and 16p13 could affect the expression of P-gp, MRP_1 and LRP in STS.

It was observed that P-gp expression is not modified by breakpoints in or loss of 7q21. However, only 5 STS could be detected from our database with breakpoints in or loss of 7q21 whereas eight cases had gain of 7q21. In combination with the frequent expression of P-gp STS (chapter 7 of this thesis) [100-103,108,297,324], this suggests that deletion of P-gp gene on 7q21 is infrequent. No evidence was found that gain of chromosomal material of 7q21 leads to more expression of P-gp.

MRP_1 expression seems not to be influenced by chromosome breakage or loss in STS, although MRP_1 expression in the 27 STS with normal chromosomes 16 was also low. Another recent study of immunohistochemically detected MRP_1 in STS, indicates that MRP_1 expression in STS is low as compared to P-gp and LRP expression (chapter 7 of this thesis). This means that the influence of chromosome breakage on immunohistochemically detected MRP_1 expression has to be examined in larger groups of specific histological types with relatively high MRP_1 expression.

LRP expression was low or absent in most of the tumors with breakpoints in or loss of 16p11, but abundant in the other STS with normal chromosomes 16. This indicates that, unlike P-gp and MRP_1, damage or loss of the 16p11 region of one chromosome could dramatically alter the expression of LRP. Furthermore, when the amount of intact chromosome 16p11 material was correlated with LRP expression, the LRP expression was more prominent in the tumors with normal chromosomes 16 or gain of 16p11. Van der Kolk *et al.* (personal communication) have observed a similar correlation between the biological activity of MRP_1 and the number of intact MRP_1 genes in AML. Since LRP expression is prominent in STS in general (chapter 7 of this thesis), a non-functioning LRP gene can be recognized easily. In myxoid LPS, LRP expression was lower than in other LPS, indicating that low LRP expression is not related to lipogenic differentiation. The importance of the chromosomal aberrations in 16p11 on the expression of LRP is further strenghtened by the finding of a t(12;16)(q13;p11) in 9 of the myxoid LPS. In parallel to studies

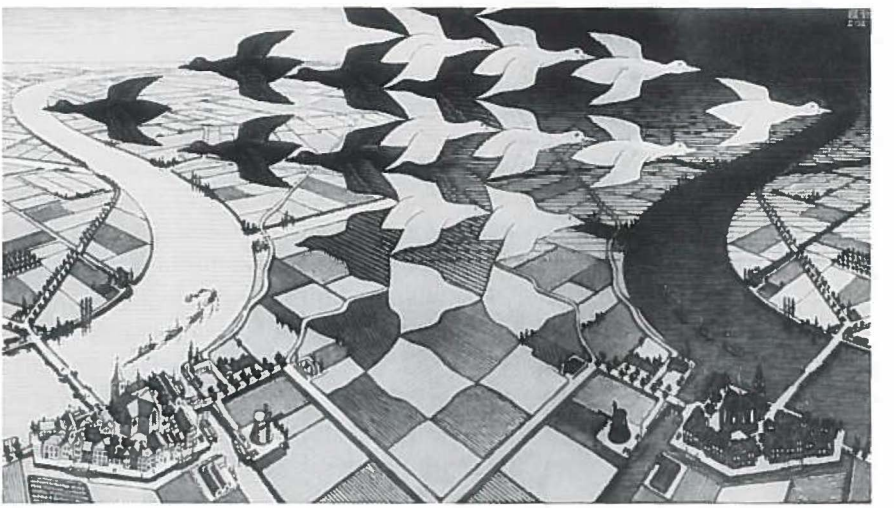
reporting a correlation between tumor grade and P-gp and MRP_1 expression, low LRP levels might also be explained by the fact that all myxoid LPS were low grade tumors [100,108,455]. However, in a previous study (chapter 7 of this thesis) no relation between LRP expression and tumor grade was found and also in the present study the expression of LRP did not correlate with tumor grade. Although LRP has been shown to be associated with clinical drug resistance in ovarian carcinoma, AML and multiple myeloma, LRP has also been detected in many normal cell types and is thought to act as an intracellular transporter protein not only carrying MDR cytotoxic substances towards the extracellular environment [95-97,292,294,296]. LRP seems to be correlated with MRP_1 expression in this group of STS which were selected for the presence of 16p11-13 abnormalities. Twelve out of 16 (75%) LRP negative tumors were also MRP_1 negative, which indicates that chromosome breakage in or loss of 16p11 could also affect the expression of the MRP_1 gene. However, of the 27 STS with normal 16p13 regions 14 cases were MRP_1 negative and 6 of 21 cases with a cytogenetically normal 16p11 had no expression of LRP. This indicates that, although a cytogenetically normal gene is present it is not necessarily transcribed. Future studies in STS, should examine the expression of and interaction between other ATP binding cassette membrane efflux pumps such as MRP_2 or MRP_6, which are of clinical importance in other malignancies, or the *ARA* (anthracycline resistance associated) gene, located immediately beside the MRP_1 gene [354,459].

Since the present study was performed with conventional cytogenetics, non-identified chromosomal material, e.g. marker chromosomes, could contain the P-gp or MRP_1 gene. Furthermore, DNA mutations or small deletions can not be recognized with cytogenetic analysis. Therefore, future studies should use FISH or spectral karyotyping to identify "hidden" 7q21 or 16p13 regions with intact P-gp or MRP_1 genes.

In conclusion, the expression of LRP in STS seems to be influenced by chromosome breakage, loss or gain, while P-gp and MRP_1 expression are not. Future FISH studies might confirm the relation between 16p11 aberrations, the expression and the biological activity of LRP in STS and the response to alkalyting cytostatic agents.

Chapter 15

CLINICAL OUTCOME OF PATIENTS WITH PREVIOUSLY UNTREATED SOFT TISSUE SARCOMAS IN RELATION TO TUMOR GRADE, DNA PLOIDY AND KARYOTYPE



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SUMMARY

The most important prognostic factor in soft tissue sarcomas (STS) is tumor grade. Since most grading methods are subject to the interpretation of the individual pathologist, there is a need for more objective criteria like DNA ploidy and karyotype, which are of prognostic value in several malignancies. The current study analyses the relationship between tumor grade, DNA ploidy, cytogenetic abnormalities and the clinical outcome of 44 previously untreated patients with 12 different histological types of primary STS. The tumors were graded according to the method described by Coindre which resulted in 9 grade I (20%), 18 grade II (41%) and 17 grade III (39%) STS. DNA flow cytometry and chromosomal analysis were performed using standard techniques. After a median follow-up time of 39 (range: 2 to 124) months, Kaplan-Meier survival analysis was performed. Significant differences in 5-years overall survival were found between patients with grade I or II and grade III STS ($p<0.05$). Seventeen STS were aneuploid and twenty-six were euploid. In 21 out of 39 successfully cultured STS an abnormal karyotype was found. There were no significant differences in survival in relation to DNA ploidy or the presence of chromosomal abnormalities. These results demonstrate that grading is of more prognostic value than DNA ploidy or the presence of cytogenetic abnormalities in this heterogeneous group of STS. Further studies on the different histological types of STS and including more patients are needed to definitely asses the prognostic value of these adjuvant techniques.

INTRODUCTION

In soft tissue sarcomas (STS) tumor grade is the most significant prognostic factor [8]. A higher tumor grade is associated with a worse prognosis. However, different grading systems are being used [29] which consider mitotic activity and necrosis as most important factors. The significance of other criteria, such as tumor type and differentiation level, cellularity, nuclear atypia, cellular pleomorphism, a-mount of fibrous stroma and extent of myxoid areas is debated. Because these factors are subject to the interpretation of the pathologist, more objective criteria are needed [29]. One such criterion could be DNA ploidy, which has been shown to be of prognostic value in different types of epithelial tumors as well as in sarcomas of bone and cartilage [40]. Previous studies in soft tissue tumors have indicated that DNA ploidy is associated with malignancy and tumor grade, i.e. benign soft tissue tumors are almost always diploid and sarcomas are either diploid or aneuploid [40]. Aneuploidy is more common in high grade sarcomas than in low- or intermediate grade sarcomas [42]. The significance of DNA ploidy as a prognostic factor, however, may differ among the various histological types of soft tissue sarcomas [156,159,160].

Previous studies on cytogenetic abnormalities in sarcomas have focused on the significance for histological classification [49,53,457]. In addition, some studies have suggested that chromosomal changes occur in the process of tumor progression [42,457].

In hematologic malignancies and in brain tumors cytogenetic abnormalities have been shown to be of prognostic value [358]. In STS few attempts have been made to establish a relationship between chromosomal abnormalities and prognosis. Recent studies showed a higher risk for relapse in patients with a malignant fibrous histiocytoma expressing 19p+ [163].

In a previous study we compared the relation between cytogenetic abnormalities and DNA ploidy of benign, borderline malignant and different grades of malignant soft tissue tumors [42]. The current study analyses the relationship between tumor grade, DNA ploidy, cytogenetic abnormalities and the clinical outcome of 44 previously untreated patients with different malignant soft tissue tumors.

MATERIALS AND METHODS

Selection of material

During the period 1984 - 1993 an attempt was made to obtain karyotypes from all sarcoma specimens submitted for pathological examination. The criteria for inclusion in the current study were 1) a histological diagnosis of a primary, malignant mesenchymal tumor, located in the soft tissues, which was previously untreated, 2) the availability of fresh, viable tissue submitted for chromosomal analysis and 3) the availability of clinical follow-up of the patients who were diagnosed at least 2 years before analysis. Mesenchymal proliferations of parenchymal organs were excluded. Cases with a doubtful histological diagnosis in regard to the mesenchymal character of the tumor were excluded, but malignant tumors with a definite mesenchymal histogenesis, but uncertain classification were included.

Patients

Between 1984 and 1993, 44 patients fulfilled the selection criteria and were studied. The patient group consisted of 18 male and 26 female patients. Age of the patient, histological diagnosis and tumor grade of all 44 cases are presented in Table I. The age of the patients at the time of diagnosis ranged from 10 to 84 years. The median age was 54 years. Four tumors were located in the head-neck region (9%), two in the thoracic region (5%), two in the abdomen (5%), three in the retroperitoneum (7%), five in the upper limb (11%) and twenty-eight in the lower limb (63%).

Disease free survival (DFS) time (i.e. the time between the treatment of the primary tumor and the time of local recurrence or distant metastasis) and overall survival (OS) time were recorded. All patient and treatment data were documented. Patients with marginal resections received adjuvant external beam radiotherapy. Patients with wide local resections or radical resections e.g. amputations received no adjuvant radiotherapy. These treatments were considered adequate. Patients with intralesional resections or contaminated surgical resections received adjuvant high dose radiotherapy, and their treatment was considered inadequate. Patients with adequate local treatment with distant metastasis at the time of diagnosis were

analyzed in the group of inadequately treated patients, since there was no curative treatment option.

Tumor parameters

The tumor parameters grade, DNA ploidy and karyotype were studied. Because other studies reported the artefact of normal karyotypes most likely due to overgrowing fibroblasts in culture [42], the results of DNA flowcytometry and chromosomal analysis were also combined by subdividing the STS in four groups: 1) DNA-euploid with a normal karyotype, 2) DNA-euploid with an abnormal karyotype, 3) DNA-aneuploid with an abnormal karyotype, 4) DNA- aneuploid with a normal or unsuccessful karyotype.

Histology and grading

In all cases the histological diagnosis was made on H&E stained paraffin sections with or without additional immunohistological stains on paraffin and/or frozen sections. All cases were classified according to Enzinger and Weiss [8] which revealed 13 different histological types (table 1). The STS were graded according to the system described by Coindre et al. [29], in which points are assigned to differentiation level (1: closely resembling normal tissue; 2: certain histogenetic classification; 3: undifferentiated), mitotic index per 2 mm^2 (1: 0-9; 2: 10-19; 3: 20 or more) and necrosis (0: none; 1: less than 50%; 2: more than 50%). Tumors with a total score of 2 or 3 were graded as grade I, those with a total score of 4 or 5 as grade II and those with a total score of 6-8 as grade III. This resulted in 9 grade I (20%), 18 grade II (41%) and 17 grade III (39%) STS.

DNA flow cytometry

DNA flow cytometry was primarily performed on single cell suspensions obtained from frozen material adjacent to the material submitted for tissue culture and chromosome analysis. If this did not yield an interpretable result, samples prepared from paraffin blocks of the same operation specimen were used. Samples from frozen material were prepared according to the method described by Vindelov [460]. Nuclear suspensions from paraffin embedded tissue were made according to method described by Hedley [461]. H&E-stained histologic slides were used to select representative parts of the tumor. To determine the DNA ploidy, isolated nuclei from the tumor were stained with propidium iodide and the intensity of the fluorescent signal was measured using a FACS 440-, FACStar- (Becton Dickinson) or ICP22- (Ortho) flow cytometer. In each sample at least 10.000 nuclei were analyzed.

The DNA profile was considered euploid when a single stem line was present in the diploid or in the tetraploid range; all others were considered aneuploid.

Tissue culture and chromosome analysis

Fresh representative tumor material for chromosomal analysis was cultured for 6-15 days in RPMI 1640 (Gibco), supplemented with 13.5% fetal calf serum, L-glutamin and penicillin/streptomycin. Cultures were harvested and chromosome

preparations were made according to standard cytogenetic techniques. The metaphases were air dried and stained with Giemsa after G banding with either trypsin 1:250 (0.25%, Difco) or pancreatin (0.1%, Sigma). The karyotypes were described according to the ISCN (1995) Guidelines for Cancer Cytogenetics [377].

Statistical analysis

In order to compare overall survival (OS) and disease free survival (DFS) for the different patient groups in relation to grade, DNA ploidy, karyotype and the combination of DNA ploidy and karyotype, actuarial survival curves were constructed by the Kaplan-Meier method. Survival curves in the different groups were compared by the log-rank test. A Chi squared test or Chi squared test for trend was used to estimate the differences in tumor characteristics (tumor grade, DNA ploidy and karyotype). A p-value of <0.05 was considered statistically significant.

RESULTS

Tumor grade in relation to DNA ploidy and karyotype

Histology: The two most frequent histological diagnoses were malignant fibrous histiocytoma (MFH; 12 cases) and myxoid liposarcoma (8 cases) (Table I). Nine tumors were grade I (20%), 18 grade II (41%) and 17 grade III (39%).

DNA ploidy: All grade I tumors except one (an extraosseous myxoid chondrosarcoma), appeared to be DNA-euploid. In contrast, 8 of the 18 grade II and 8 of the 17 grade III tumors were DNA-aneuploid (Table II). The suspension of one grade III MFH yielded no interpretable results. The difference in distribution of the euploid and aneuploid tumors among the three tumor grade groups was not quite statistically significant ($p= 0.08$). There were significantly more aneuploid MFHs than aneuploid liposarcomas or synovial sarcomas: 2 out of 13 liposarcomas and 1 out of 6 synovial sarcomas were aneuploid, whereas 9 out of 13 MFHs were aneuploid ($p<0.05$).

Karyotype: Abnormal karyotypes were found in 4 out of 9 grade I (44%), 10 out of 18 grade II (56%) and 7 of 17 grade III (41%) tumors (Table II). In 8 liposarcomas, 4 synovial sarcomas and in 4 out of 12 MFHs an abnormal karyotype was found. The differences among these groups were not statistically significant.

Table 1. Patients, tumor grade, histology and median age

tumor grade	histologic diagnosis	n	age (years) median
grade I (n=9)	myxoid liposarcoma	5	49
	synovial sarcoma	1	25
	clear cell sarcoma	1	32
	MFH[1]	1	74
	extraosseous myxoid chondrosarcoma	1	84
grade II (n=18)	synovial sarcoma	5	49
	rhabdomyosarcoma	3	22
	non-myxoid liposarcoma	3	68
	MFH[1]	3	57
	myxoid liposarcoma	2	32
	leiomyosarcoma	2	62
grade III (n=17)	MFH[1]	8	71
	malignant rhabdoid tumor	3	10
	non-myxoid liposarcoma	2	60
	myxoid liposarcoma	1	50
	fibrosarcoma	1	21
	pPNET[2]	1	56
	alveolar soft part sarcoma	1	58

[1]MFH: malignant fibrous histiocytoma; [2]pPNET: peripheral primitive neuroectodermal tumor

Table 2. Patients, tumor grade, DNA ploidy and karyotype

Tumor grade	DNA ploidy	N	Karyotype	n
Grade I (n=9)	Euploid	8	Normal	3
			abnormal	4
			unsuccessful	1
	aneuploid	1	unsuccessful	1
Grade II (n=18)	euploid	10	normal	2
			abnormal	6
			unsuccessful	2
	aneuploid	8	normal	2
			abnormal	4
			unsuccessful	2
Grade III (n=17)	euploid	8	normal	4
			abnormal	4
	aneuploid	8	normal	4
			abnormal	3
			unsuccessful	1
	unsuccessful	1	normal	1

Combination of karyotype and DNA ploidy: There were 9 euploid tumors with a normal karyotype, 14 euploid tumors with an abnormal karyotype, 7 aneuploid tumors with an abnormal karyotype. The remaining group of 14 STS consisted of aneuploid tumors with either a normal or unsuccessful karyotype or in which assessment of DNA ploidy or karyotyping was unsuccessful. Euploid STS with an abnormal karyotype can be explained by either minor numerical or structural chromosomal aberrations as discussed before [42,462].

Treatment

Of the 44 patients 33 were treated for cure. Nine other patients were treated for local cure but had distant metastases at the time of diagnosis. In the remaining two other patients adequate treatment was not conceivable since surgery or adjuvant radiotherapy was not possible because of the location of the tumor (1 patient) or adjuvant radiotherapy was not desirable because of poor wound healing (1 patient).

Overall survival and disease free survival

The median follow-up period was 39 months and ranged from 2 months in a woman who died from a grade III malignant rhabdoid tumor, to 124 months, a man with a grade II leiomyosarcoma. Survival status in relation to tumor grade, DNA ploidy and karyotype is presented in Table III. At the end of the follow-up period 18 patients were alive with no evidence of disease (NED), 2 patients were alive with disease (AWD) and 19 patients died of their disease (DOD). Two patients died of another cause after 84 and 11 months follow-up. Three patients were lost to follow-up. Five patients had an isolated local recurrence, 9 patients developed isolated distant metastases and 4 patients developed a local recurrence with distant metastases. Fifteen out of 19 patients developed a local recurrence or metastasis within 2 years after treatment. In the patients with distant metastases DNA flowcytometry of their primary tumor revealed 8 euploid and 5 aneuploid patterns.

Table 3. Survival status in relation to tumor grade, DNA ploidy and karyotype

Survival status	number of patients	Grade			DNA-ploidy			Karyotype		
		I	II	III	Eupl.	Aneupl.	Unsucc.	Normal	Abnormal	Unsucc.
NED	18	4	10	4	10	8	0	5	11	2
AWD	2	1	1	0	2	0	0	1	1	0
DOD	19	3	5	11	12	6	1	9	7	3
DOC	2	0	0	2	1	1	0	1	1	0
LOFU	3	1	2	0	1	2	0	0	1	2
total	44	9	18	17	26	17	1	16	21	7

NED: alive, no evidence of disease; AWD: alive with disease; DOD: died of disease; DOC: died of other causes; LOFU: Lost to follow-up; Eupl.: euploid, Aneupl.: aneuploid; Unsucc.: unsuccessful

OS and DFS of STS-patients were determined in relation to tumor grade, DNA ploidy, karyotype and the four groups in which karyotype and DNA ploidy were combined. In view of the limited number of patients, a 2-years DFS analysis was performed. Table IV shows the mean and median OS and DFS time as well as the 5-years OS and 2-years DFS percentages.

OS curves according to the different tumor grades are shown in Figure 1. Significant differences in OS time were found between patients with grade I and grade III STS ($p = 0.02$) as well as between patients with grade II and grade III STS ($p = 0.005$). OS analysis revealed neither significant differences between patients with DNA-euploid versus -aneuploid STS (Fig. 2) nor between patients with STS with a normal versus abnormal karyotype (Fig. 3) . Furthermore, no significant differences in OS were observed among the four patient groups in which the results of DNA flowcytometry and chromosomal analysis were combined (Fig. 4). The differences in 2-years DFS of the analyzed patient groups were not statistically significant.

After stratification for adequate and inadequate treatment or for the three major histological types (MFH, liposarcoma, synovial sarcoma), no significant differences in OS and DFS were found in relation to DNA ploidy and karyotype. A significant difference ($p<0.0001$) in OS was found between the patients who were adequately treated (mean OS time: 56 months) and those who were not (mean OS time: 20 months). The 5-years OS of patients which were adequately treated was 75%, while the 5-years OS of patients who could not be adequately treated was 9%.

Table 4.
Overall and disease free survival according to tumor grade, DNA ploidy and karyotype

parameter	number of patients	OS (months) mean/median	DFS (months) mean/median	5-yrs OS	2-yrs DFS
grade I	8	93 / 111	71 / 50	75 %	63 %
grade II	16	93 / 108	78 / -	74 %	75 %
grade III	17	42 / 16 *	54 / 21	29 %*	46 %
euploid STS	25	73 / 108	68 / 50	59 %	68 %
aneuploid STS	15	80 / -	78 / -	56 %	58 %
normal karyotype	16	58 / 31	46 / 36	47 %	55 %
abnormal karyotype	20	85 / -	75 / -	64 %	67 %
combination groups					
euploid + normal karyotype	9	54 / 31	35 / 36	43 %	58 %
euploid + abnormal karyotype	14	83 / -	80 / -	63 %	76 %
aneuploid + abnormal karyotype	6	79 / -	60 / 21	67 %	50 %
aneuploid + normal or unsuccessful karyotype	12	72/111	75 / -	55 %	56 %

* Statistically significant ($p < 0.05$) difference between grade I/II and III STS.

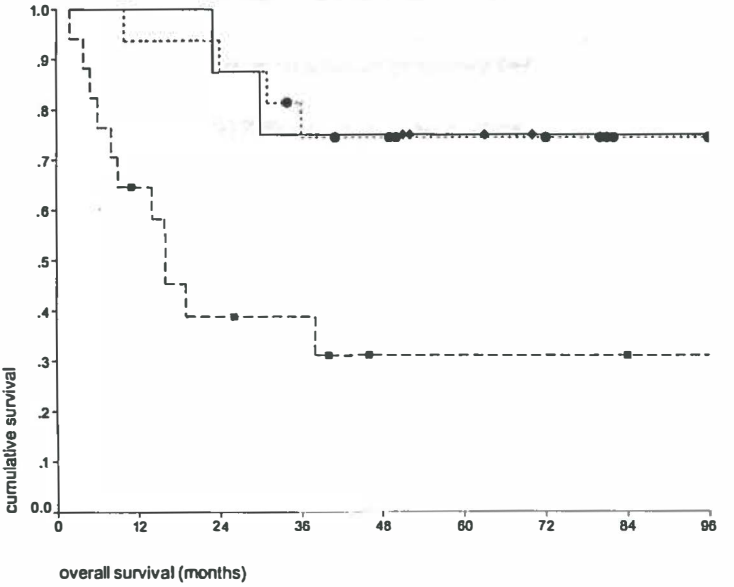


Figure 1
Overall survival for patients with grade I (◆), grade II (●) and grade III (■) STS.

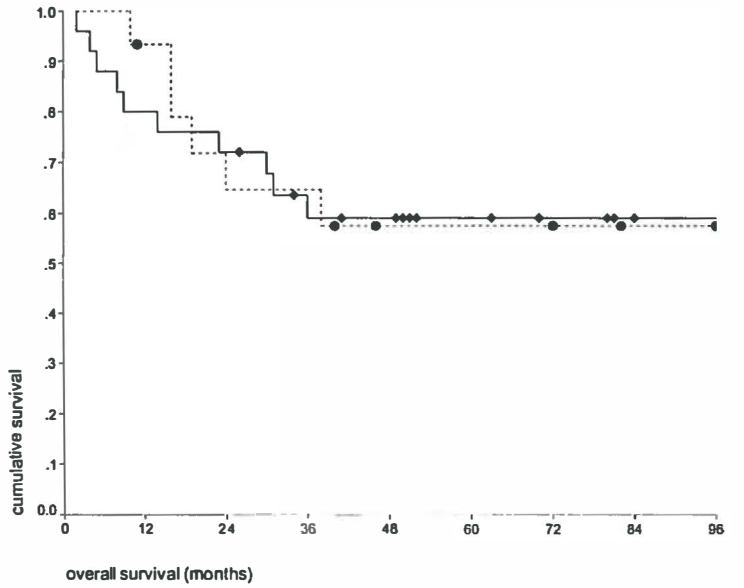


Figure 2
Overall survival for patients with euploid (◆) and aneuploid (●) STS

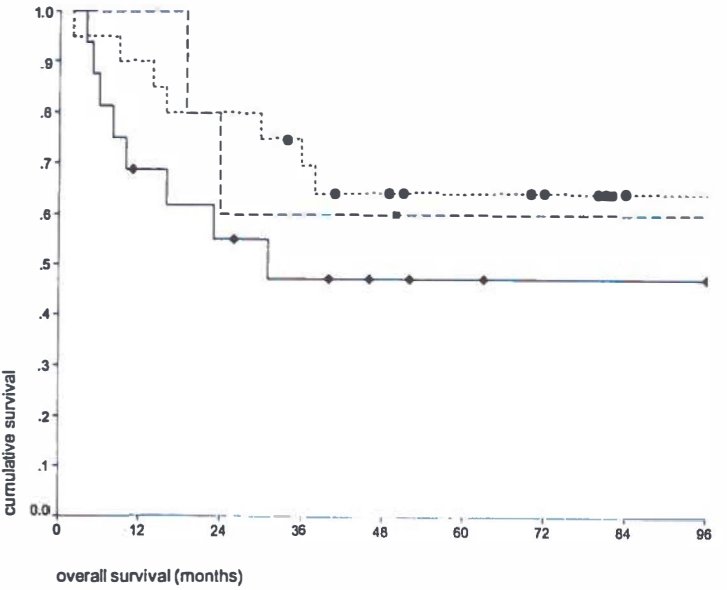


Figure 3
Overall survival for patients with STS expressing a normal (◆) or abnormal (●) karyotype or in which karyotyping was unsuccessful (■).

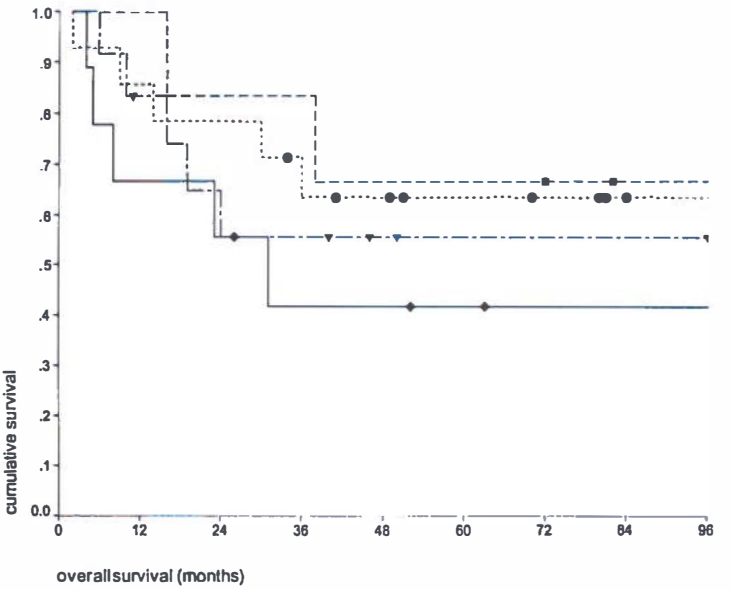


Figure 4
Overall survival for the four STS-patient groups with euploid and cytogenetically normal STS (◆), euploid and cytogenetically abnormal STS (●), aneuploid and cytogenetically abnormal STS (■) and the remaining STS expressing either an aneuploid STS with a normal karyotype or in which assessment of DNA ploidy or karyotyping was unsuccessful (▼).

DISCUSSION

The prognosis of patients with STS is determined by three major factors: the patient, the tumor (histology and stage) and the treatment. In order to adjust therapy to improve survival of a patient, the pathologist has to estimate the degree of malignancy by assessing the biological properties of the tumor. Therefore several systems of classification and grading have been established for STS. Because these systems are subject to controversies [29], there is a need for more objective criteria which are directly related to the individual tumor and have a high prognostic value. The prognostic value of two such possible criteria (DNA ploidy and karyotype) was assessed in relation to the established grading system as described by Coindre [29] on 44 patients with histotypically different STS. To reduce the possibility of therapy induced alterations in DNA content or chromosomal aberrations, only previously untreated patients were entered in this study.

Tumor grade, DNA ploidy and karyotype

The majority of the liposarcomas and synovial sarcomas were euploid and had a structural or minor numerical chromosomal aberration. In contrast, the majority of MFHs were aneuploid, but had a normal karyotype. Half of the grade III STS were aneuploid, while in grade I STS only 1 out of 9 was aneuploid. Half of all grade III STS revealed a normal karyotype, while only 4 out 18 grade II STS and 3 out of 9 grade I STS did. This relative high percentage of normal karyotypes in grade III STS and MFH together with a tendency towards DNA-aneuploidy when tumor grade is increasing, could be explained by assuming that normal fibroblasts had an *in vitro* growth advantage [42].

Overall survival and disease free survival

By determining OS and DFS of STS-patients with different tumor grades, we showed that our patient group was representative. In concordance with reports in the literature, we found a significant relationship between tumor grade and survival [8].

No statistically significant relationship was found between DNA ploidy and survival which is in agreement with recent studies of heterogeneous groups of STS [126,157]. In contrast, in other studies a relationship was found between DNA ploidy and prognosis, i.e. DNA-aneuploid tumors had a worse prognosis than euploid tumors [156]. However, studies of specific histological types of STS revealed different outcomes [156,159,160]. For example in synovial sarcomas a relationship was found between DNA ploidy and survival [159], whereas in liposarcomas DNA ploidy was not of prognostic value [160]. The lack of a correlation between DNA ploidy and survival in our group could be explained by the fact that our group of STS included relatively many liposarcomas.

Karyotyping has been shown to be an adjunct in histological diagnosis. Whether it is of prognostic significance has to be determined. In this respect, we believe that the finding of an abnormal karyotype (e.g. t(12;16)(q13;p11) in myxoid or round cell liposarcoma) in a primary untreated STS is of more diagnostic value

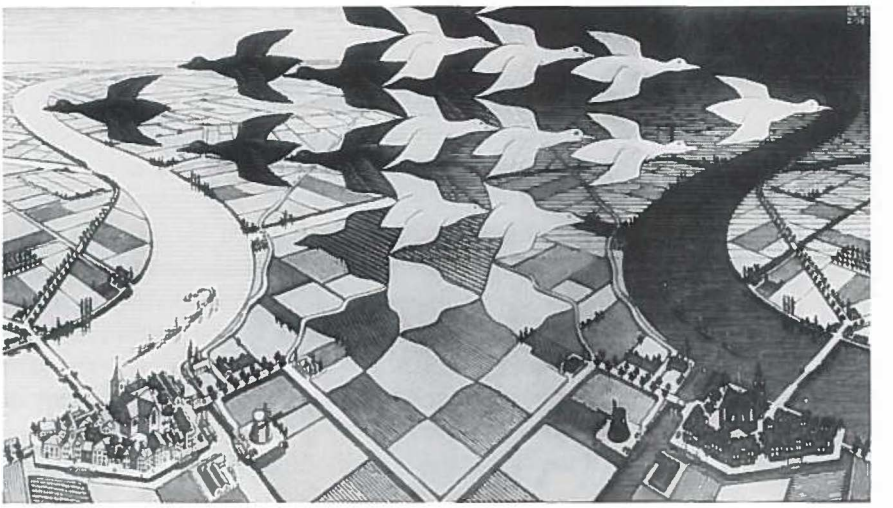
than it is of prognostic value. Additional chromosomal abnormalities could be of more prognostic value than the finding of a type characteristic cytogenetic anomaly [55,463]. Comparing the cytogenetic results with survival, no statistically significant differences were found between patients with cytogenetically normal or abnormal STS. This could be due to the previous described difficulties in culturing highly malignant STS [42]. Even by the formation of 4 groups combining DNA ploidy and karyotype in order to solve this problem of the overgrowing fibroblasts, no significant differences in OS and DFS were found between those groups. Recent studies [163] showed that chromosomal alterations involving 19p13 are associated with a high recurrence rate in MFH. This was not confirmed in our study, in which one MFH showing an alteration in 19p13 was found in a patient who is without evidence of disease 105 months after treatment.

This study shows that for this heterogeneous group of previously untreated patients with STS, the histological grade of the primary tumor is of more prognostic value than DNA ploidy and the karyotypic findings. Further studies in larger groups of untreated patients, focusing on separate histological types of STS are mandatory. In this way the prognostic value of these techniques can further be assessed.

Acknowledgment
We are grateful to Dr.Ir. G.J. te Meerman for his advice in statistical analysis.

Chapter 16

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES



The heterogeneous group of STS is characterized by their unknown tumorigenesis and the absence of clear tumor biological prognostic markers. Traditional prognostic factors such as tumor grade and size still have the best predictive value. Research has been performed on tumor biological characteristics which could be helpful in the adequate diagnosis of STS, tumor tailored therapy and the prediction of treatment results and clinical outcome. Although the knowledge of oncogenesis and treatment induced biological changes in STS is increasing, many questions have still to be answered.

In this thesis, histopathological and cytogenetic methods in STS were used to assess their value in diagnosis, treatment and the prediction of clinical outcome.

HISTOPATHOLOGY

A reliable recognition of mitotic figures needs training.

Tumor grade is one of the most important prognostic factors in STS. An inaccurate assessment of tumor grade may also have clinical consequences. In most of the commonly used grading systems, the mitotic activity plays a central role. It is known that there is interobserver variability in the assessment of mitotic figures and that the number of mitoses also depends on the fixation protocol. Another method to assess proliferative activity is the MIB-1 labeling of proliferating cells. MIB-1 is an antibody directed against the nuclear antigen Ki-67. Those two methods were compared in **chapter 2.** It was demonstrated that experience in the assessment of mitotic figures decreases the "unexplained" variance in the mitotic counts. The assessment of MIB-1 detected proliferation is less influenced by experience and the "unexplained" variance is lower than in the mitotic counts. However, staining costs are relatively high as compared to the simple and quick haematoxylin-eosin staining used in the counting of mitotic figures. MIB-1 detected proliferation and mitotic indices were not correlated, indicating that proliferating cells are not the same as dividing cells: Ki-67 precedes the actual cell division and remains present in the nucleus even when the cell cycle is interrupted.

This study suggests that the assessment of mitotic figures is a learning process which can be trained. Modern image software and multimedia devices, could be used for this purpose in the training of pathologists. This will be helpful in the differentiation from, for instance, apoptotic cells. It will further improve the internal personal and laboratory standard for the recognition of mitotic figures and will lead to a more uniform assessment of malignancy grade.

TYR-PET is a suitable way to assess proliferative activity in STS.

As has been demonstrated in **chapter 3**, the proliferative activity in STS can also be detected *in vivo* by the assessment of protein synthesis rate (PSR) using positron emission tomography with L-[1-^{11}C]tyrosine (TYR-PET). A correlation was found between PSR and the Ki-67 proliferation index, and between PSR and mitotic rate.

These findings indicate that the non-invasive method of TYR-PET can estimate the mitotic and proliferative activity in STS and might provide additional information in the assessment of malignancy grade. It can be used in the detection of those tumor regions that have the highest proliferative activity. This would be helpful in the determination of the optimal localization for tumor biopsy. In addition it might be worthwhile to use TYR-PET in the evaluation of treatment responses in *vivo*, which could be more reliable that the traditional assessment with physical examination and radiographical screening.

IMT-SPECT can also be used to assess proliferative activity in STS.

Another method to visualize PSR and amino acid transport in tumors is the use of single photon emission computerized tomography (SPECT) with ^{123}I-labeled amino acid L-3-[^{123}I] iodo-alpha-methyl tyrosine (IMT). Proliferative and mitotic activity is a measure of malignancy grade and can be assessed *in vitro*. Cellularity has also been used in grading systems and could affect the tumor related PSR. Furthermore, the amount of blood vessels and capillaries in a tumor could also affect the PSR specific IMT-SPECT signal. In **chapter 4** of this thesis, it was found that in benign and malignant soft tissue tumors IMT-SPECT correlates with cellularity, proliferative and mitotic activity , but not with vascularity.

This means that IMT-SPECT is indeed a measure for malignancy *in vivo* and could be used to detect proliferative activity in tumors. These results indicate that IMT-SPECT could be used for detection of high proliferative regions before diagnostic surgery or could be helpful in the assessment of treatment response. Unlike TYR-PET, IMT-SPECT does not require a complex infrastructure. However, in future studies IMT-SPECT should be validated and compared with TYR-PET in a group of solely malignant soft tissue tumors.

Response to HILP with TNF-α and melphalan is better in STS with high mitotic activity and is associated with an increase in apoptotic cells.

Hyperthermic isolated limb perfusion with tumor necrosis factor-α (TNF-α) and melphalan (HILP-TM) with or without interferon-γ (IFN-γ) is a breakthrough in the local treatment of patients with primary irresectable locally advanced extremity STS with response rates of up to 84%. The mechanisms of the treatment response are poorly understood. **Chapter 5** of this thesis demonstrates that proliferative activity dropped after HILP-TM, whereas the amount of apoptosis was increased. The addition of IFN-γ to HILP-TM did not influence the changes in tumor parameters and did not affect treatment response. A better clinical response to HILP-TM was correlated with high mitotic activity and low amount of apoptosis in tumor samples before HILP-TM. Patients with highly proliferative STS before and after HILP-TM had a relatively poor prognosis. Furthermore, patients who developed distant metastases after HILP-TM had a relatively high number of dividing cells in the residual tumor after treatment.

These results indicate that HILP-TM directly affects tumor cells and that tumor biological processes involved in apoptosis are related with the response rate in HILP-TM treated patients with locally advanced STS. The value of other proteins involved in the apoptotic pathway for the prediction of treatment response has to be evaluated in future studies.

Proliferative activity after HILP with TNF-α and melphalan can be measured with TYR-PET.

In the experimental treatment with HILP-TM a period of 6-8 weeks is used before delayed local excision of the perfused residual tumor will take place. In **chapter 6** it has been demonstrated that the proliferative and mitotic activity before HILP-TM can be measured with both ^{18}F-fluoro-deoxy-D-glucose (FDG)-PET, a method to visualize glucose consumption, and TYR-PET. However, after HILP-TM, the correlation between FDG-PET and proliferative activity disappeared, whereas correlation between proliferative activity and TYR-PET improved.

This means that TYR-PET is the most suitable tracer to assess responses to HILP-TM. This is important since with TYR-PET the turning point in proliferative activity in the perfused tumor can be visualized and the optimal time point for delayed tumor resection can be chosen.

Expression of P-gp, MRP$_1$ and LRP varies between the histological types of STS.

In case of diffuse distant metastases, chemotherapy is used in STS. Poor responses to chemotherapy are observed in specific types of STS, especially leiomyosarcoma (LMS) and malignant fibrous histiocytoma (MFH). Liposarcomas (LPS) have a favorable response to chemotherapy. Cross resistance to a number of functionally and structurally distinct natural product drugs is called multidrug resistance (MDR). Several mechanisms have been found to be responsible for MDR. In this study we have focussed on expression of P-glycoprotein (P-gp), the multidrug resistance related protein-1 (MRP$_1$) and the lung resistance protein (LRP). In **chapter 7** of this thesis, a series of 115 chemotherapy naive STS was studied for the expression of P-gp, MRP$_1$ and LRP with respect to histological type and tumor grade. Seventy-one percent of the STS expressed P-gp, 36% MRP$_1$ and 70% LRP. At least one MDR protein was detected in 95% of the STS. No correlation was found between tumor grade and the expression of P-gp, MRP$_1$ or LRP. However, co-expression was less frequently observed in low grade STS. These results demonstrate that P-gp, MRP$_1$ and LRP are expressed in the majority of STS, which could explain their poor responses to chemotherapy. Expression of P-gp, MRP$_1$ and LRP varies between histological types and is not related to tumor grade. LPS, known for their relatively good response to chemotherapy, did show remarkably low LRP levels, which might be due to the high proportion of myxoid LPS with a 16p11 breakpoint (chapter 13). The in the literature reported poor responses in leiomyosarcomas were not reflected by abundant P-gp, MRP$_1$ or LRP expression in the examined soft tissue LMS.

Further studies have to evaluate the prognostic value of the (co)-expression of these parameters in relation with various treatment regimens in each of the histological types.

LMS and GIST differ in clinical outcome, metastatic pattern and MDR.

Several studies have reported clinical behavior and drug resistance in leiomyosarcomas, but did not differentiate between soft tissue LMS and malignant gastrointestinal stromal tumors (GIST). Multidrug resistance (MDR) has been associated with the expression of P-gp (P-glycoprotein), MRP_1 (multidrug resistance protein) and LRP (lung resistance protein). In **chapter 8**, the differences between LMS and GIST with respect to clinical outcome and MDR parameters were studied. The mean overall survival in patients with a LMS was 72 months and 31 months in patients with a GIST ($p<0.05$). Metastases occurred in 53% of the GIST patients and in 58% of the LMS patients. In GIST patients with known metastatic disease during follow-up, liver metastases were found in 5 patients and intra-abdominal metastases in 3 patients. The lungs were the primary affected metastatic site in 13 (87%) of the metastasized LMS and in none of the GIST patients ($P<0.001$), whereas subsequent liver metastases developed in 4 LMS patients. P-gp and MRP_1 expression was significantly ($p<0.05$) more pronounced in GIST than in LMS, i.e. 31% of the LMS and 68% of the GIST were P-gp positive and 31% of the LMS and 68% of the GIST were MRP_1 positive. These findings demonstrate that LMS patients have a better survival than GIST patients. Although the frequency of distant metastases in LMS and GIST are comparable, LMS predominantly metastasize to the lungs, while GIST tend to spread to the liver and the abdominal cavity.

LMS have a less pronounced drug resistance pattern than GIST. Future studies should therefore differentiate between LMS of soft tissue and GIST. These findings indicate that previously studied clinical and histopathological variables "leiomyosarcomas" have to be re-evaluated. Studies reporting a high number of gastro-intestinal leiomyosarcomas, without differentiation, have to be interpreted with extreme caution. Therefore, studies are needed to establish the clinical, histopathological and (onco)genetic differences between LMS and GIST, especially in relation with their metastastatic behavior and their response to chemotherapy.

P-gp, MRP_1 and LRP expression cannot predict responses to EVI polychemotherapy in STS.

A large proportion of adult patients with disseminated STS do not respond to chemotherapy which might be due to multidrug resistance (MDR) protein expression. MDR has been associated with the expression of P-gp, MRP_1 and LRP. Whereas P-gp and MRP_1 are involved in the efflux of anthracyclines (doxorubicin, epirubicin), the exact role of LRP in the efflux of cytostatic agents is less known. In **chapter 9** of this thesis, we studied 28 adult STS patients with metastatic disease or irresectable tumor, treated with epirubicin, vindesine and ifosfamide (EVI) for treatment response and evaluated the expression of P-gp, MRP_1 and LRP with respect to treatment response and clinical outcome. Fifty-eight percent of the STS

were P-gp positive, 61% were MRP_1 positive and 68% were LRP positive. In 93% of the STS at least one analyzed MDR-protein was present. Co-expression was found in 75% of the tumors. MDR-protein expression was not associated with treatment response. Overall and progression free survival (OS and PFS) were not associated with the expression of P-gp, MRP_1 or LRP. However, patients with sarcomas co-expressing two or three drug resistance proteins had a worse median PFS (7 months) than those without co-expression (11 months). These results indicate that, although the majority of adult STS expresses P-gp, MRP_1 and/or LRP, the assessment of P-gp, MRP_1 or LRP expression in the individual patient with a metastasized STS cannot predict response to EVI treatment. However, co-expression of P-gp, MRP_1 and/or LRP tends to be associated with poor PFS.

These findings indicate that expression of single MDR proteins in STS alone can not predict responses to chemotherapy and implies that additional tumor biological parameters have to be evaluated. Furthermore, multi-center studies in larger, uniformly treated, patient groups are needed to validate the results of this study.

(CYTO)GENETICS

(Cyto)genetic analysis is helpful in clarifying oncogenesis of various malignant solid tumors and the observation of recurrent chromosome abnormalities led to the detection of tumor specific chromosomal alterations which are of diagnostic value. Characteristic cytogenetic alterations have been found in several histologic types of STS. However, most of the published studies in small groups of tumors presented karyotypes in such a way that even shared chromosomal alterations are difficult to detect and a lot of cytogenetic information could not be used in this way for a direct comparison of specific tumors. This problem of interpreting and comparing different tumor groups was the reason for the construction of the database which was used in chapters 10 and 11 of this thesis. This direct comparison of cytogenetic data and the visualization in so called karyographs makes it easier to interpret the differences between groups of tumors e.g. malignant peripheral nerve sheath tumors (MPNST), leiomyosarcomas (LMS) or gastrointestinal stromal tumors (GIST). This method of computer assisted conventional cytogenetics gives the opportunity to expand the understanding of the cytogenetic anomalies of uncommon solid tumors by taking together all cytogenetic studies of small numbers of cases and extract meaningful shared cytogenetic aberrations.

Significant loss of 9p2 and gain of 7q1 in MPNST might be of oncogenetic importance.

Cytogenetic studies in small groups of patients with malignant peripheral nerve sheath tumors (MPNST) revealed complex karyotypes without consistent changes. In **chapter 10**, a computer assisted cytogenetic analysis using a cytogenetic

database was performed, to determine recurrent cytogenetic alterations in 51 MPNSTs and to assess differences between sporadic MPNST and neurofibromatosis-1 associated MPNST. Significant ($p<0.05$) loss was observed in the chromosomal regions 9p2, 11p1, 11q2 and 18p1. Gain of chromosomal material was found in chromosome 7, especially 7q1 ($p<0.05$). Cytogenetic differences between NF-1 associated and sporadic MPNSTs included a relative loss of chromosomal material in NF-1 associated MPNSTs in 1p3, 4p1 and 21p1-q2 and a relative gain in 15p1-q1. This approach, in which the cytogenetic results of various reports are combined, shows that losses in 9p2 and gains in 7q1 could be of oncogenetic importance in MPNSTs. Loss of 17q1, on which the NF-1 gene has been located (17q11.2), is not a common cytogenetic finding in NF-1 associated MPNSTs. The observed differences between NF-1 associated and sporadic MPNSTs might reflect different oncogenetic pathways.

Further studies in MPNST should focus on the genes located on the chromosomal regions with significant gains or losses. The expression of for instance the p16 protein is expected to be low as compared to STS without loss of 9p2. The use of so called computer assisted cytogenetic analysis is not a substitution of conventional cytogenetics. It is merely a tool to obtain more information from already cytogenetically analyzed tumors. In this way karyotypes can be interpreted more easily and a comparison between specific patient or tumor groups can be made. In the next future, this method can be used to compare metastases and their primary tumors and might contribute to the detection of those tumors with high metastatic potential.

Cytogenetic comparison of GIST, LMS and MPNST.

The histogenesis, oncogenesis and clinical behavior of malignant gastrointestinal stromal tumors (GIST) are the subject of dispute, since these neoplasms may reveal either neural differentiation, resembling malignant peripheral nerve sheath tumors (MPNST), or smooth muscle differentiation, resembling leiomyosarcomas of deep soft tissue (LMS). Previous cytogenetic studies did not clearly differentiate between GIST, LMS and MPNST. In **chapter 11**, the earlier mentioned computer assisted method of direct cytogenetic comparison was used to discover tumor specific chromosomal patterns in 16 malignant mesenchymal tumors ("GIST") of the digestive tract, 14 soft tissue LMS and 37 MPNST. In "GIST" a statistically significant loss was observed in chromosomal regions 13q2-q3, 14p1-14q2, 18p1-18q2, 22p1 and 22q1. In LMS the chromosomal changes did not reach statistical significance. However, important loss was found in 5p1, 14p1 and of chromosome 22, whereas gain was observed of chromosome X. In MPNST a significant loss of chromosomal material was detected in 9p2, 11p1, 11q2 and 18p1, as well as important loss in 22q; a significant gain was noticed in the 7q1 region. This cytogenetic meta-analysis supports the hypothesis that malignant mesenchymal tumors arising in the digestive tract or "GIST" are a distinct entity which differs from LMS and MPNST and is characterized by losses in 13q2-q3, 14p1-q2, 18p1-q2 and 22p1-q1. However, the shared loss of chromosome 22 in "GIST", LMS and MPNST,

might be indicative for a shared oncogenetic pathway, whereas gain of 7q1 and loss of 18p1-q2 seems to be more associated with neural differentiation.

This study did not succeed to discover chromosomal aberrations which could be of highly diagnostic importance. However, differences in these small groups of already cytogenetically analyzed tumors make further evaluations worthwhile. These studies should use a combination of CGH for detection of more specific chromosomal alteration, molecular genetics for detection of *c-kit* mutations and immunohistochemistry to detect more significant differences.

(Cyto)genetic analyses in STS can have clinical consequences.

In **chapter 12**, a case is described of a boy with a small cell tumor with a cytogenetically discovered t(11;22). Molecular genetic analysis revealed also a EWS-FLI-1 fusion transcript, which has also been observed in Ewing's sarcoma. After excisional biopsy, the boy was treated with Ewing's sarcoma based chemotherapy and is still without evidence of disease three years after treatment. This case is an example that additional (cyto)genetic data might have clinical consequences.

The detection of fusion transcripts and their future immunohistological assessment, are of utmost diagnostic and clinical importance.

The 16p11.2 breakpoint in myxoid liposarcomas might affect the expression of the LRP gene on 16p11.2.

Myxoid liposarcomas (LPS) are characterized by the t(12;16)(q13;p11) which leads to the formation of a FUS-CHOP fusion transcript. In **chapter 13** of this thesis, we examined the relationship between the cytogenetically detected breakpoint 16p11 in myxoid LPS, the presence of the FUS-CHOP fusion transcript in non-myxoid LPS and the expression of the lung resistance major vault protein (LRP) gene on 16p11.2. In all 9 myxoid LPS a t(12;16)(q13;p11) was found and LRP expression was absent or low. In none of the remaining 7 cases of a with a preferential diagnosis of liposarcoma a FUS-CHOP fusion transcript was found and four tumors were LRP positive ($p=0.02$).

These observations indicate a relation between the t(12;16)(q13;p11), leading to a FUS-CHOP fusion transcript in myxoid LPS, and the low or absent expression of the LRP-gene located on 16p11.2. Further studies have to examine whether LRP expression is related to the breakpoint 16p11 characteristic for myxoid LPS. It would be interesting to investigate if myxoid LPS have better response to drugs which are normally affected by LRP, e.g. melphalan in HILP-TM. The relation between chromosomal aberrations and the expression of MDR proteins was further investigated in chapter 14.

Chromosomal aberrations in 16p11 might affect LRP expression in STS.

Multidrug resistance (MDR) has been associated with the expression of the P-gp (P-glycoprotein), LRP (lung resistance protein) and MRP_1 (multidrug resistance related protein) genes on chromosomes 7q21, 16p11 and 16p13. Chromosome

breakage in 16p13 could influence the expression of MRP_1, as has been shown in specific cases of acute myeloid leukemia. In soft tissue sarcomas (STS), cytogenetic analyses revealed characteristic cytogenetic alterations in several histologic types. The influence of chromosomal aberrations on protein expression has not been studied before in STS. In **chapter 14** of this thesis, it was found that P-gp and MRP_1 expression did not differ when STS with breakpoint in or loss of the 7q21 or 16p13 region were compared to STS with normal 7q21 or 16p13 regions. However, in STS with breakpoints in or loss of 16p11, the median percentage of LRP expressing cells was 3% and in STS with cytogenetically intact 16p11 regions the median expression was 40% ($p<0.01$). LRP expression, but not MRP_1 or P-gp expression, correlated with the amount of cytogenetically intact chromosomal material. LRP expression in liposarcomas did not differ from STS other than liposarcomas, whereas myxoid liposarcomas had lower LRP expression than other liposarcomas ($p<0.01$), indicating that low LRP expression is not associated with the lipomatous character but with the 16p11 breakpoint (as observed in chapter 13). We conclude that cytogenetically detected loss or breakpoints in 16p11 might affect LRP expression, whereas P-gp and MRP_1 expression are not clearly affected by loss of or breakpoints in the chromosomal regions 7q21 and 16p13, respectively.

This study indicates that in STS the MDR associated protein LRP plays a prominent role: it is abundantly expressed in STS, but is low in STS with damage of 16p11 material. The possible association between the reported favorable response rates in advanced (myxoid) LPS, the 16p11 breakpoint, LRP expression and the various forms of chemotherapy or HILP with TNF-α and melphalan have to be investigated.

Abnormal karyotypes and DNA-aneuploidy are not of prognostic value in STS.

DNA ploidy and karyotype could of prognostic value in STS. In **chapter 15**, we examined the relationship between tumor grade, DNA ploidy, cytogenetic abnormalities and the clinical outcome of 44 previously untreated patients with 12 different histological types of primary STS. Significant differences in 5-years overall survival were found between patients with grade I or II and grade III STS ($p<0.05$). Seventeen STS were aneuploid and twenty-six were euploid. In 21 out of 39 successfully cultured STS an abnormal karyotype was found. There were no significant differences in survival in relation to DNA ploidy or the presence of chromosomal abnormalities.

These results demonstrate that grading is of more prognostic value than DNA ploidy or the presence of an abnormal karyotype. The use of other (cytogenetic) methods such as CGH or computer assisted cytogenetic analyses have to compare different homogeneous groups, e.g. MFH and LMS or patient groups with a favorable and poor clinical outcome. In this way more specific chromosomal regions could be identified which can have predictive value for tumor progression or treatment response.

General conclusions and future perspectives:

The work described in this thesis is focussed on the relation of certain tumor biological parameters (i.e. proliferation, apoptosis, multidrug resistance, DNA-ploidy and chromosomal aberrations) with the histopathological diagnosis, treatment response and clinical outcome of patients with a STS.

The diagnosis of STS can be difficult and tumor grading is influenced by the experience of the individual pathologist. New imaging techniques such as TYR-PET and IMT-SPECT can support the diagnosis and the assessment of the tumor grade, but further studies on tumor biology related radionuclides are needed. The additional diagnostic value of conventional cytogenetics has reached its limits and other cytogenetic methods such as computer assisted cytogenetics or CGH will provide useful diagnostic information. The results of the described computer assisted cytogenetic studies in MPNST and GIST revealed chromosomal changes with could be of additional diagnostic value. Other histological types should be compared with the same method to discover "hidden" chromosomal differences within the various STS. It is expected that especially molecular genetic techniques in combination with histopathological examination, i.e. the immunohistological detection of fusion transcripts such as the FUS-CHOP or the EWS-FLI-1 protein, will have additional diagnostic value.

One of the objectives of this thesis was to determine the influence of proliferative activity or apoptosis on the response to HILP-TM in locally advanced primary STS. We found that proliferative activity and apoptosis of tumor cells might influence the spectacular responses to HILP-TM, whereas HILP-TM appears to alter the proliferative and apoptotic activity in the tumors. Future studies on the expression of recently detected cell cycle and apoptosis related genes/proteins in combination with examination of the endothelial changes after HILP-TH might reveal tumor related predictive factors. With the improvement of culturing techniques, *in vitro* studies on tumor cells and the vasculature in short term cultures of STS could clarify the biological mechanism of HILP-TM. Tumor biological examination of the residual tumor after HILP-TM could elucidate the biological resistance to HILP-TM. When tumor markers are studied in relation to treatment response, the assessment of those responses have to be highly reliable. Tumor biology related imaging techniques such as TYR-PET or IMT-SPECT turned out to provide tumor proliferation related information which might be more specific than physical examination and traditional radiographic screening according to 20-years old WHO standards. It was shown that chemotherapeutic treatment of advanced/metastasized STS with epirubicin, vindesine and ifosfamide results in a promising response rate of 59%. The expression of the single MDR proteins P-gp, MRP_1 and LRP can not predict treatment response or clinical outcome. However, the expression of these MDR-proteins and their distribution within the various histological types is now better understood. The relation between (cyto)genetics and histopathology has been emphasized with the observed relation between 16p11 aberrations and decreased LRP expression. Future

studies in LPS have to examine the clinical relation: do myxoid LPS or other STS with 16p11 aberrations respond better to alkalyting agents containing chemotherapeutic regimens? This thesis weakens the conclusions of many clinical studies that LMS are characterized by liver metastases, drug resistance and poor survival. We demonstrate that previous studies in "leiomyosarcomas" examined the biological behavior of both LMS and GIST. LMS do not frequently metastasize to the liver and have low P-gp and MRP_1 levels as compared to GIST. Survival in patients with GIST is relatively poor, but not associated with MDR expression.

In the examined specific patient groups, only tumor grade, mitotic activity and proliferation had some prognostic value . Other parameters such as the presence of an abnormal karyotype, DNA-aneuploidy, expression of single MDR proteins and amount of apoptosis, could not be identified as prognostic markers. Taken together, treatment response and clinical outcome are influenced by many biological parameters. It would be narrow minded to assume that only one or two biological variables would affect the metastatic potential or clinical outcome of an individual STS. The combination of predictive markers, rather than single parameters, might provide useful prognostic information. In the next future, studies in large homogeneous groups of STS have to validate recently discovered tumor biological parameters. New techniques such as DNA-chip technology could be helpful: in this way a pattern of risk factors for each individual STS can be developed, which might reveal clinically important information such as the prediction of treatment response or the occurrence of distant metastases. Because of the rarity of the whole group of STS, this can only be done in large collaborative studies. Furthermore, future studies in STS should concentrate on distant metastases. It should be determined whether expression of proteins involved in tumor proliferation, apoptosis, and angiogenesis or the expression of adhesion molecules could predict development of metastases. Conventional (computer assisted) cytogenetics and comparative genomic hybridization might reveal which chromosomal parts are involved in the metastatic process This might lead to early detection of STS with high metastatic potential. To predict the MDR pattern of the distant metastases by examination of the primary tumor, evaluation of the changes in expression of MDR-proteins between primary tumors and their distant metastases is necessary. Further studies in uniformly treated patients should investigate the (co-)expression of other drug resistance related proteins and genes/proteins involved in the processes leading to apoptosis.

With the progress of new insights in tumor biology, it is a challenge for the next future to search for new diagnostic and prognostic factors which can be helpful in histopathological diagnosis of STS and will lead to more tumor adjusted therapies for patients with STS.

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NEDERLANDSE SAMENVATTING

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Weke delen sarcomen (WDS) zijn kwaadaardige (maligne) tumoren die ontstaan uit de mesenchymale cellen die o.a. het bindweefsel, vetweefsel en spierweefsel vormen. Het zijn zeldzame tumoren die slechts 1% van alle maligniteiten vormen. Afhankelijk van hun uitrijpingskenmerken (differentiatie) wordt deze heterogene groep tumoren onderverdeeld in o.a. liposarcomen (LPS), leiomyosarcomen (LMS), rhabdomyosarcomen, maligne fibreus histiocytomen (MFH), maligne perifere zenuwschede tumoren (of MPNST) en synoviosarcomen. De onstaanswijze van WDS is onduidelijk. De reactie op verschillende vormen van behandeling, zoals bijvoorbeeld chemotherapie of geïsoleerde ledemaat perfusie met TNF-α en melphalan (HILP-TM), is niet goed te voorspellen. Wel is uit eerder onderzoek gebleken dat een hoge mate van kwaadaardige kenmerken (hoge maligniteitsgraad) en een grote tumor het klinisch beloop (de overleving) negatief beïnvloeden.

De laatste jaren is steeds meer bekend geworden over de invloed van bepaalde biologische mechanismen in tumorcellen op verschillende behandelingen en op het ziekte beloop. Binnen de groep van WDS is echter in het verleden relatief weinig onderzoek verricht naar tumorcel gerelateerde biologische eigenschappen, die behulpzaam zouden kunnen zijn bij het stellen van een adequate diagnose of het voorspellen van de behandelingsresultaten en het ziektebeloop.

In het onderzoek dat beschreven wordt in dit proefschrift, wordt onderzocht of bepaalde histopathologische en cytogenetische parameters kunnen bijdragen aan het stellen van de diagnose, de behandelingsresultaten kunnen beïnvloeden en het klinisch beloop van bepaalde patiëntengroepen kunnen voorspellen.

Hiervoor zijn verschillende methoden gebruikt en is gekeken naar een aantal eigenschappen van een kwaadaardige tumor.

Wanneer een tumor veel kenmerken heeft van een kwaadaardige tumor, zoals veel celdelingen (*mitosen*), veel necrose en een slechte differentiatie, wordt gesproken van een hoge maligniteitsgraad of *tumorgraad*. Niet alleen het aantal delende cellen, maar ook de hoeveelheid cellen die zich klaarmaken zijn om te gaan delen (*prolifereren*) kan informatie geven over de agressiviteit van een tumor. Deze prolifererende cellen werden in dit onderzoek aangetoond met behulp van een zogenaamde immuunhistochemische kleuring waarbij een bepaald antilichaam (MIB-1) het Ki-67 eiwit in de kern herkent. Ki-67 is een eiwit wat alleen voorkomt in de kernen van cellen die aan het prolifereren zijn. Wanneer een "gezonde" cel door beschadiging van het erfelijke materiaal (het DNA) een tumorcel dreigt te worden zal deze zichzelf vernietigen. Dit proces van zelfvernietiging heet geprogrammeerde celdood of "*apoptose*". Cellen die zichzelf vernietigen hebben bepaalde aan apoptose gerelateerde kenmerken die o.a. kunnen worden aangetoond met behulp van een speciale kleuringstechniek, de TUNEL- methode. Bepaalde behandelingen zoals het geven van cytostatica of bestraling hebben juist het doel om nog meer DNA-schade te veroorzaken zodat tumorcellen uiteindelijk toch in apoptose zullen gaan en de

tumor dus dood gaat. Wanneer een tumor niet dood gaat na het geven van chemotherapie, kan dit ook veroorzaakt worden doordat een tumor als het ware resistent is tegen de verschillende cytostatica. Nu is de laatste jaren duidelijk geworden dat hierbij een aantal eiwitten een rol spelen die het effect van chemotherapie tegenwerken, wat "*multidrug resistance*" (MDR) wordt genoemd. In de celmembraan zitten o.a. twee soorten pompen die de cytostatica de tumorcel uit kunnen pompen: het P-glycoprotein (*P-gp*) en het multidrug resistance protein-1 (*MRP_1*). P-gp en MRP_1 zijn betrokken bij o.a. het uitpompen van anthracyclines (doxorubicine, epirubicine), cytostatica die veelvuldig gebruikt worden bij de behandeling van WDS. Een ander eiwit wat zich met name in het cytoplasma bevindt en schadelijke, alkylerende stoffen de cel uit lijkt te transporteren wordt het lung resistance protein (*LRP*) genoemd. De exacte rol van LRP in de efflux van cytostatica is niet goed bekend. Deze drie eiwitten werden in dit onderzoek aangetoond met behulp van immunhistochemische kleuringen.

In de meeste maligne tumoren zijn allerlei afwijkingen in het DNA ontstaan: er ontbreken stukken DNA of er is extra DNA gemaakt. De hoeveelheid DNA (*DNA-ploidie*) in een cel kan gemeten worden met behulp van DNA-flowcytometrie. Hierbij wordt het DNA gekleurd met een fluorescerende stof, waarna men de cellen door een dun buisje laat stromen en ze worden beschenen met een laser-straal. De hoeveelheid gereflecteerd licht wordt gemeten en is dan een maat voor de hoeveelheid DNA.

Een andere manier om naar veranderingen in het DNA te kijken is door *chromosomen* te bestuderen (*cytogenetica*). Vlak voor een cel gaat delen zal het DNA samentrekken tot 23 paar chromosomen, waarbij een vrouw twee X chromosomen heeft en een man een X chromosoom en een Y chromosoom in de lichaamscellen heeft. Chromosomen kunnen zichtbaar gemaakt worden op microscopisch niveau (1000x vergroot) tijdens bepaalde stadia van de celdeling. Ze hebben een korte en een lange arm (p en q arm) gescheiden door een centromeer in het midden van het chromosoom. Met behulp van speciale kleuringstechnieken vertoont elk chromosoom een uniek patroon van bandjes, waaraan de individuele chromosomen kunnen worden herkend. De verschillende chromosoombanden zijn genummerd volgens internationaal geldende regels. Op grond van deze kenmerken kunnen afwijkingen van het normale patroon herkend en beschreven worden. Het is bekend dat bij veel WDS karakteristieke chromosomale afwijkingen voorkomen die gebruikt kunnen worden bij het stellen van de juiste diagnose. In veel WDS zijn er echter allerlei verschillende chromosomale afwijkingen.

HISTOPATHOLOGIE

Voor een betrouwbare herkenning van mitosen is ervaring nodig.

Tumorgraad is een van de belangrijkste prognostische factoren in WDS. Een verkeerde beoordeling van de tumorgraad kan klinische consequenties hebben. Bij de meeste graderingsmethoden speelt het tellen van het aantal celdelingsfiguren (mitosen) een centrale rol, waarbij een zekere subjectiviteit van de beoordelende patholoog een rol kan spelen. In **hoofdstuk 2** werden twee methoden gebruikt die beide te maken hebben met celdeling: het beoordelen van het aantal mitosen en de hoeveelheid prolifererende cellen. Er werd aangetoond dat ervaring in het beoordelen van mitosen de "onverklaarde" statistische spreiding verlaagt. Hoewel de beoordeling van MIB-1 gedetecteerde proliferatie minder werd beïnvloed door het verschil in ervaring, zijn de kosten van deze methode veel hoger en is een correlatie tussen MIB-1 detectie en mitose frequentie niet altijd aanwezig. Daarom wordt geadviseerd om de ervaring in het herkennen van mitosefiguren, en het onderscheid met bijvoorbeeld apoptose, te vergroten door middel van training. Hiervoor zouden moderne interactieve multimedia-technieken gebruikt kunnen worden binnen de opleiding tot patholoog, waardoor de interne persoonlijke standaard en de laboratoriumstandaard zouden kunnen verbeteren en maligniteitsgraad op een uniformere manier kan worden bepaald.

TYR-PET is een bruikbare manier om proliferatie in WDS te meten.

De eiwitsynthese van tumoren, wat een maat is voor de biologische activiteit, kan worden bepaald door middel van positron emissie tomografie met L-[1-^{11}C]tyrosine (TYR-PET). L-[1-^{11}C]tyrosine is een radioactief gemaakt aminozuur wat in eiwitten wordt ingebouwd. De hoeveelheid gemeten radioactiviteit is dan een maat voor de eiwitsynthese. In **hoofdstuk 3** werd een relatie gevonden tussen de eiwitsynthese gemeten met TYR-PET en zowel de mitosefrequentie als de Ki-67 proliferatie index. Dit impliceert dat TYR-PET niet alleen kan bijdragen aan de beoordeling van de maligniteitsgraad, maar ook een rol kan spelen bij het bepalen van die tumor regio's met de hoogste proliferatie. Dit zou van belang kunnen zijn in het bepalen van de optimale biopsieplaats. Verder zou TYR-PET gebruikt kunnen worden in de evaluatie van behandelingsresponsen *in vivo*, hetgeen misschien bruikbaarder zou kunnen zijn dan de huidige beoordeling door middel van lichamelijk onderzoek en radiologische screening.

IMT-SPECT kan ook worden gebruikt om proliferatie in WDS te beoordelen.

Een andere methode om eiwitsynthese in tumoren te beoordelen is single photon emission computerized tomography (SPECT) met radioactief (^{123}I-gelabeled) aminozuur L-3-[^{123}I] iodo-alpha-methyl tyrosine (IMT). In **hoofdstuk 4** wordt beschreven dat in een groep van zowel goedaardige als kwaadaardige weke delen tumoren de mate van het IMT-SPECT signaal correleert met zowel de celdichtheid in een tumorbiopt als met de mate van proliferatie en mitosefrequentie. Dit betekent dat met behulp van IMT-SPECT maligniteit *in vivo* kan worden beoordeeld en dat deze

methode de mate van proliferatie zou kunnen bepalen. In tegenstelling tot TYR-PET, is voor de IMT-SPECT methode niet een complexe infrastructuur nodig. Echter, in toekomstige studies zal IMT-SPECT moeten worden vergeleken met TYR-PET in een groep die bestaat uit alleen maligne weke delen tumoren (WDS).

Response op HILP met TNF-α en melphalan is gerelateerd aan de mitosefrequentie en gaat gepaard met een toename van apoptotische cellen.

Hyperthermische geïsoleerde ledemaat perfusie met tumor necrose factor-α (TNF-α) en melphalan (HILP-TM) met of zonder interferon-γ (IFN-γ) kan worden beschouwd als een doorbraak in de behandeling van patiënten met niet chirurgisch verwijderbare grote WDS van de extremiteiten. Het mechanisme achter de behandelingsrespons is nog steeds onduidelijk, hoewel gedacht wordt dat TNF-α en melphalan o.a. een beschadiging geeft van de bloedvaten die de tumor van bloed voorzien, waarna uiteindelijk door stolselvorming de bloedvaten verstoppen en de tumor dood gaat. **Hoofdstuk 5** van dit proefschrift laat zien dat de mate van proliferatie afneemt na HILP-TM, terwijl de hoeveelheid apoptotische cellen toeneemt. De toevoeging van IFN-γ aan HILP-TM beïnvloedde de veranderingen in de onderzochte tumorparameters overigens niet en had ook geen effect op de behandelingsrespons. Wel leek een betere klinische response na HILP-TM gecorreleerd aan hoge mitose activiteit en lage hoeveelheid apoptose in de tumoren, zoals beoordeeld voor HILP-TM. Patiënten met sterk prolifererende WDS, zowel voor als na HILP-TM, hadden een relatief slechte prognose. Patiënten die metastasen ontwikkelden na HILP-TM hadden een relatief hoge delingsactiviteit in de overgebleven tumor resten na HILP-TM. Deze resultaten wijzen erop dat HILP-TM ook op een direct wijze de tumorcellen beïnvloedt en dat biologische processen binnen het apoptose mechanisme betrokken zouden kunnen zijn bij de respons op HILP-TM.

Proliferatie na HILP met TNF-α en melphalan kan worden gemeten met TYR-PET.

In de experimentele behandeling van WDS met HILP-TM is het gebruikelijk om na een periode van 6-8 weken de tumorresten alsnog chirurgisch te verwijderen. In **hoofdstuk 6** wordt in twee groepen patiënten aangetoond dat proliferatie- en mitose-activiteit voor het starten van HILP-TM kan worden gemeten met behulp van zowel ^{18}F-fluoro-deoxy-D-glucose (FDG)-PET, een methode om glucose consumptie wat een maat is voor de biologische activiteit (suikerstofwisseling), als TYR-PET. Echter, na HILP-TM verdween in de ene groep de correlatie tussen FDG-PET en proliferatie, terwijl in de andere groep de correlatie tussen tumorcelproliferatie en TYR-PET verbeterde. Dit duidt erop dat TYR-PET de best bruikbare PET-methode is om responsen op HILP-TM te beoordelen. Dit is belangrijk omdat nu met TYR-PET de response van de geperfundeerde tumor kan worden beoordeeld en op indicatie het moment voor verwijderen van de tumorresten kan worden bepaald.

Expressie van P-gp, MRP_1 en LRP varieert tussen de histologische typen van WDS.

In het geval van (diffuse) uitzaaiingen (metastasen) is chemotherapie geïndiceerd. Hoewel in het algemeen slechts 20-40% van de WDS reageert op cytostatica, lijken vooral leiomyosarcomen (LMS) en maligne fibreus histiocytomen (MFH) slecht te reageren. Liposarcomen (LPS) hebben daarentegen een relatief goede reactie op chemotherapie. De studie beschreven in **hoofdstuk 7** werd 115 WDS, die niet eerder behandeld waren met chemotherapie, de expressie van P-gp, MRP_1 en LRP bestudeerd in relatie tot het histologische type en de tumor graad. De resultaten laten zien deze MDR-eiwitten tot expressie worden gebracht in het merendeel van de WDS, wat mogelijk de in het algemeen slechte respons op chemotherapie kan verklaren. De expressie van P-gp, MRP_1 en LRP varieert tussen histologische types en is niet duidelijk gerelateerd aan tumorgraad. LPS, die goed reageren op chemotherapie, hadden opmerkelijk weinig LRP expressie, hetgeen mogelijk verklaard zou kunnen worden door het relatief hoge aandeel myxoid LPS met hun karakteristiek breukpunt in chromosoom 16p11 (hoofdstuk 13). Opvallend was dat LMS, waarvan in de literatuur de relatief slechte respons op chemotherapie wordt benadrukt, een relatief lage expressie hadden van P-gp en MRP_1. Verder onderzoek is noodzakelijk om prognostische waarde van (co)-expressie van MDR eiwitten te evalueren en een relatie te leggen met de verschillende combinaties van cytostatica voor elk van de histologische types.

LMS en GIST verschillen in klinisch beloop, metastaserings patroon en MDR.

In verscheidene klinische studies werd met name een hoge mate van chemotherapieresistentie in leiomyosarcomen beschreven. Echter, de meeste onderzoeken maakten geen onderscheid tussen "echte" weke delen LMS en gastrointestinale stroma tumoren (GIST), die in het verleden ook wel leiomyosarcomen van het maag-darm kanaal werden genoemd. In **hoofdstuk 8**, werden de verschillen tussen LMS en GIST ten aanzien van het klinisch beloop en expressie van MDR eiwitten onderzocht. Er werd aangetoond dat LMS patiënten een betere prognose hebben dan GIST patiënten. Hoewel de frequentie van afstandsmetastasen in LMS en GIST vergelijkbaar is, metastaseren LMS voornamelijk naar de longen, terwijl GIST lijken uit te zaaien naar de lever en de buikholte. Expressie van P-gp en MRP_1 was minder uitgesproken in LMS dan in GIST. Toekomstige studies moeten daarom een duidelijk onderscheid maken tussen weke delen LMS en GIST. Dit alles wijst erop dat de conclusies van eerdere klinische en histopathologische studies in "leiomyosarcomen" opnieuw moeten worden geëvalueerd. In de toekomst zijn studies nodig die zich richten op de klinische, histopathologische en (onco)genetische verschillen tussen LMS en GIST, met name in relatie met hun metastaseringsgedrag en de reactie op chemotherapie.

De expressie van P-gp, MRP_1 en LRP kan de reactie op EVI polychemotherapie in STS niet voorspellen.

Een aanzienlijk deel van de volwassen patiënten met een uitgezaaid WDS reageert onvoldoende op chemotherapie wat mogelijk wordt veroorzaakt door expressie van P-gp, MRP_1 en LRP. In **hoofdstuk 9** van dit proefschrift, werden 28 volwassen WDS patiënten, met afstandsmetastasen of niet chirurgisch te verwijderen tumor, behandeld met epirubicine, vindesine en ifosfamide (EVI) en werd de relatie tussen de expressie van P-gp, MRP_1 en LRP bestudeerd in relatie met de behandelingrespons en het klinisch beloop. Hoewel het merendeel van de WDS MDR-eiwitten tot expressie brengt, de beoordeling hiervan in het geval van de individuele patiënt met een gemetastaseerd WDS de respons op behandeling met EVI niet kan voorspellen. Echter, co-expressie van P-gp, MRP_1 en/of LRP lijkt mogelijk te zijn gerelateerd aan een slechtere progressie vrije overleving. Deze resultaten impliceren dat het beoordelen van alleen de expressie van MDR eiwitten in WDS niet voldoende is om respons op chemotherapie te voorspellen en dat additionele tumor biologische parameters moeten worden onderzocht. Verder dient onderzocht te worden of de expressie van de verschillende MDR-eiwitten verband houdt met het biologisch gedrag van de WDS.

(CYTO)GENETICA

In de meeste gepubliceerde cytogenetische studies van relatief kleine groepen tumoren werden de karyotypen op zodanige wijze gepresenteerd dat bepaalde overeenkomstige chromosomale afwijkingen moeilijk te detecteren waren en een relatief grote hoeveelheid aan waardevolle cytogenetische informatie niet kon worden gebruikt in de vergelijking tussen verschillende groepen tumoren. Dit probleem van interpretatie en vergelijking van tumor groepen was de reden voor het construeren van een cytogenetische database die werd gebruikt in de hoofdstukken 10 en 11.

Significant verlies van 9p2 en winst van 7q1 in MPNST kan belangrijk zijn in de oncogenese.

Cytogenetische studies in kleine groepen patiënten met een maligne zenuwschede tumor of "malignant peripheral nerve sheath tumor" (MPNST) leverden complexe karyotypen op zonder aanwijzingen voor consistente chromosomale veranderingen. In **hoofdstuk 10,** werd een zogenaamde "computer assisted cytogenetic analysis" uitgevoerd waarbij een cytogenetische database werd gebruikt om steeds terugkerende cytogenetisch veranderingen op te sporen in 51 MPNSTs en om te beoordelen of er verschillen waren tussen sporadische MPNST en neurofibromatose-1 gerelateerde MPNST. Deze benadering, waarin de cytogenetische bevindingen van verscheidene studies zijn gecombineerd, laat zien dat verlies in 9p2 en winst in 7q1 mogelijk belangrijk zou kunnen zijn in de oncogenese van MPNSTs. Verlies van 17q1, waarop het NF-1 gen is gelokaliseerd

(17q11.2), wordt niet vaak gevonden in NF-1 gerelateerde MPNSTs. De gevonden verschillen, hoewel niet statistisch significant, zouden kunnen wijzen op verschillen in oncogenese tussen NF-1 gerelateerde en sporadische MPNSTs. Vervolgstudies in MPNST zouden zich moeten richten op de genen gelokaliseerd op die chromosomale regio's met significant winst of verlies. Het is bijvoorbeeld te verwachten dat de expressie van p16, een eiwit wat de processen die leiden tot een celdeling remt, relatief laag is vergeleken met andere WDS zonder verlies van 9p2. Het gebruik van deze zogenoemde "computer assisted cytogenetic analysis" is geen substitutie van de conventionele cytogenetische analyses, maar een hulpmiddel om meer informatie te verkrijgen uit al bestaande karyotypen.

Cytogenetische vergelijking van GIST, LMS en MPNST.

De histogenese, de oncogenese en het klinisch gedrag van maligne gastrointestinale stroma tumoren (GIST) is onvoldoende opgehelderd, omdat deze neoplasmata zowel kenmerken vertonen van neurale differentiatie, wat hen doet gelijken op maligne zenuwschede tumoren (malignant peripheral nerve sheath tumors) (MPNST), als van differentiatie in de richting van glad spierweefsel, wat hen doet gelijken op leiomyosarcomen van de weke delen (LMS). Eerdere cytogenetische studies hebben geen duidelijk onderscheid gemaakt tussen GIST, LMS en MPNST. In **hoofdstuk 11**, wordt de eerder genoemde "computer assisted" methode gebruikt om eventueel specifieke verschillen in chromosomale patronen te ontdekken bij 16 malignant mesenchymale tumoren van het maag-darm kanaal ("GIST"), 14 weke delen LMS en 37 MPNST. Deze cytogenetische meta-analyse lijkt de hypothese te bevestigen dat de maligne mesenchymale tumor van het maag-darm kanaal (of wel "GIST") een aparte entiteit is die verschilt van LMS en MPNST en wordt gekarakteriseerd door verlies van 13q2-q3, 14p1-q2, 18p1-q2 en 22p1-q1. Echter, het gezamenlijke verlies van chromosoom 22 in zowel "GIST", LMS en MPNST zou kunnen wijzen op een samenhang in de oncogenese, terwijl winst van 7q1 en verlies van 18p1-q2 mogelijk gerelateerd lijkt te zijn aan een neurale differentiatie. In deze studie is het niet gelukt om chromosomale afwijkingen te ontdekken die van duidelijk diagnostische betekenis kunnen zijn. Echter, de gevonden verschillen in deze kleine groepen zouden kunnen dienen als basis voor vervolgstudies. Om in deze studies meer verschillen tussen de drie tumorgroepen op te kunnen sporen zou bijvoorbeeld "comparative genomic hybridization" (CGH) voor de detectie van specifieke kleinere chromosomale afwijkingen gecombineerd kunnen worden met zowel moleculaire genetica, voor de detectie van *c-kit* mutaties, als immunohistochemie.

(Cyto)genetische analyses in WDS kunnen klinische consequenties hebben.

In **hoofdstuk 12** wordt een casus beschreven van een jongen met een kleincellige tumor waarin bij cytogenetisch onderzoek een t(11;22) werd gevonden. Moleculair genetisch onderzoek liet verder een EWS-FLI-1 fusie transcript zien, zoals ook wordt gevonden in Ewing's sarcoom. Na excisie, werd de jongen behandeld met chemotherapie zoals voor Ewing's sarcoom en is drie jaar na diagnose nog steeds ziektevrij.

De detectie van fusietranscripten en toekomstige hierop gebaseerde immunohistologische technieken zijn van diagnostisch en klinisch belang in de toekomst.

Het 16p11.2 breukpunt in myxoide liposarcomen zou de expressie van het LRP gen op 16p11.2 kunnen beïnvloeden.

Myxoide liposarcomen (LPS) worden gekarakteriseerd door de t(12;16)(q13;p11) die leidt tot de vorming van het FUS-CHOP fusie transcript. In **hoofdstuk 13** van dit proefschrift, hebben we de relatie onderzocht tussen het cytogenetisch gedetecteerde breukpunt 16p11 in myxoide LPS, de aanwezigheid van het FUS-CHOP fusie transcript in niet-myxoide LPS en de expressie van het LRP gen op 16p11.2. In alle 9 myxoide LPS werd een t(12;16)(q13;p11) gevonden en de LRP expressie was afwezig of zeer laag. In geen van de overige 7 gevallen met een (voorkeurs)diagnose van liposarcoom werd een FUS-CHOP fusie transcript aangetroffen en 4 van de 7 tumoren waren LRP positief. Deze waarnemingen wijzen op een mogelijke relatie tussen de t(12;16)(q13;p11), die leidt tot het FUS-CHOP fusie transcript in myxoide LPS, en de lage of afwezige expressie van het LRP-gen op 16p11.2. Toekomstige studies zouden moeten onderzoeken of de expressie van LRP is gerelateerd aan het breukpunt 16p11. Het zou interessant zijn om te onderzoeken of myxoide LPS een betere respons hebben op alkylerende cytostatica waarvan de effectiviteit door de aanwezigheid van LRP mogelijk wordt beïnvloed. De relatie tussen chromosomale afwijkingen en de expressie van de MDR eiwitten wordt verder onderzocht in hoofdstuk 14.

Chromosomale afwijkingen in 16p11 kan de LRP expressie in WDS beïnvloeden.

Multidrug resistance (MDR) is gerelateerd aan de expressie van P-gp (P-glycoprotein), LRP (lung resistance protein) en MRP_1 (multidrug resistance protein) genen op chromosomen 7q21, 16p11 en 16p13. Een breuk van het chromosoom in 16p13 kan de expressie van MRP_1 beïnvloeden zoals ook is gevonden in bepaalde gevallen van acute myeloide leukemie. Het effect van chromosomale afwijkingen op eiwit expressie is nog niet eerder onderzocht in WDS. In **hoofdstuk 14** van dit proefschrift, werd gevonden dat P-gp en MRP_1 expressie niet verschilde tussen WDS met een breukpunt in of verlies van de 7q21 of de 16p13 regio wanneer zij werden vergeleken met WDS met normale 7q21 of 16p13 regio's. Echter, in WDS met breukpunten in of verlies van 16p11, was het mediane percentage cellen wat LRP tot expressie bracht 3% terwijl dit in STS met cytogenetisch intacte 16p11 regio's 40% was ($p<0.01$). Wij concluderen dat het cytogenetisch gevonden verlies van of breukpunten in 16p11 mogelijk effect heeft op de expressie van LRP, terwijl P-gp en MRP_1 expressie niet duidelijk worden beïnvloed door verlies van of breukpunten in respectievelijk de chromosomale regio's 7q21 en 16p13. Toekomstige studies zullen met moleculair genetische technieken deze resultaten moeten bevestigen.

Een abnormaal karyotype en DNA-aneuploidie zijn niet van prognostische waarde WDS.

DNA-ploidie en de aanwezigheid van chromosomale afwijkingen in tumorcellen zouden van prognostische waarde kunnen zijn in WDS. In **hoofdstuk 15**, hebben we de relatie onderzocht tussen tumor graad, DNA-ploidie, cytogenetische afwijkingen en het klinisch beloop van 44, eerder onbehandelde, patiënten met 12 verschillende typen van primaire WDS. De resultaten lieten zien dat de tumorgraad meer prognostische waarde heeft dan de DNA-ploidie of het aanwezig zijn van chromosomale afwijkingen. Het gebruik van andere (cyto)genetische methoden zoals CGH of "computer assisted cytogenetic analysis" in het vergelijken van specifieke patiëntengroepen met bijvoorbeeld een goede of slechte prognose of goede of slechte respons op chemotherapie, zou specifiekere chromosomale regio's kunnen detecteren die van belang kunnen zijn in het voorspellen van het klinisch beloop of de reactie op verschillende therapievormen.

Algemene conclusies en vooruitblik

Het werk dat wordt beschreven in dit proefschrift richt zich met name op de relatie tussen bepaalde tumorbiologische parameters (zoals proliferatie, apoptose, MDR, DNA-ploidie en chromosomale afwijkingen) en de histopathologische diagnose, behandelingsrespons en het klinisch beloop van patiënten met een WDS.
Het is bekend dat de diagnose van een WDS moeilijk kan zijn en het blijkt dat gradering van een WDS kan worden beïnvloed door het ervaringsniveau van de individuele patholoog. Nieuwe beeldvormende technieken zoals TYR-PET en IMT-SPECT kunnen behulpzaam zijn bij de diagnose en de beoordeling van de tumorgraad, maar vervolgonderzoek naar het gebruik van tumorbiologisch gerelateerde radionucliden is nodig. De diagnostische waarde van conventionele cytogenetica lijkt zijn grenzen bereikt te hebben en andere (cyto)genetische technieken zoals "computer assisted cytogenetics" of CGH zullen mogelijk meer bruikbare informatie opleveren. Naast de beschreven resultaten in LMS, MPNST en GIST kunnen in de toekomst ook andere histologische typen op deze manier worden vergeleken waardoor "verborgen" chromosomale verschillen worden ontdekt tussen de verscheidene WDS typen. Verder ligt het in de lijn der verwachting dat met name moleculair genetische technieken, zoals het aantonen van fusietranscripten, eventueel in combinatie met hierop gebaseerde immunohistochemische methoden hun waarde zullen bewijzen in de diagnostiek van WDS.

Een van de doelen van dit proefschrift was om een relatie aan te tonen tussen de hoeveelheid proliferatie of apoptose in een WDS en de reactie van de tumor op HILP-TM. Wij vonden dat tumorcelproliferatie en -apoptose de reactie op HILP-TM lijkt te beïnvloeden en HILP-TM zelf ook effect lijkt te hebben op mechanismen in de tumorcel die betrokken zijn bij proliferatie en apoptose. Binnen het onderzoek naar deze behandeling zullen in de toekomst studies zich moeten richten op de verschillende eiwitten die betrokken zijn bij apoptose en proliferatie. Wanneer tumormerkers worden bestudeerd in relatie tot de behandelingsrespons, dan moeten de methoden van beoordeling van deze respons betrouwbaar zijn. Tumorbiologie

gerelateerde beeldvormende technieken zoals TYR-PET of IMT-SPECT, die informatie over tumorproliferatie blijken te geven, zouden misschien beter de behandelingsrespons kunnen weergeven dan de combinatie van lichamelijk onderzoek en radiologische screening die gebaseerd is op 20 jaar oude WHO standaards. In dit proefschrift werd aangetoond dat cytostatische behandeling van WDS met epirubicine, vindesine en ifosfamide resulteerde in een veelbelovende respons van 59%. De expressie van de afzonderlijke MDR eiwitten P-gp, MRP_1 en LRP kan echter de behandelingsrespons of het klinisch beloop niet voorspellen in de onderzochte groep patiënten. De expressie van deze MDR-eiwitten en de verdeling over de verschillende histologische typen is door dit onderzoek nu wel beter beschreven. De relatie tussen (cyto)genetische afwijkingen en histopathologie werd benadrukt met de aangetoonde relatie tussen de afwijkingen in 16p11 en de verminderde expressie van LRP. In de toekomst zullen studies in liposarcomen de relatie met de kliniek moeten onderzoeken: hebben myxoide LPS of andere WDS met een 16p11 afwijking een betere respons op de door LRP beïnvloede alkylerende cytostatica? Dit proefschrift zwakt de conclusies af van vele klinische studies die ten onrechte beweren dat leiomyosarcomen worden gekarakteriseerd door het veelvuldig optreden van levermetastasen, het aanwezig zijn van "drugresistentie" en een extreem slechte prognose. Hieruit blijkt dat eerdere studies in groepen patiënten met "leiomyosarcomen" hoogstwaarschijnlijk het klinisch en biologisch gedrag hebben onderzocht van zowel LMS als gastrointestinale stroma tumoren (GIST), die vroeger leiomyosarcomen van het maag-darm kanaal werden genoemd.

In de onderzochte specifiek patiëntengroepen waren alleen tumorgraad, mitose activiteit en proliferatie van prognostische waarde. Andere parameters zoals het aanwezig zijn van een abnormaal karyotype, DNA-aneuploidie, de expressie van de afzonderlijke MDR eiwitten en de mate van apoptose, konden niet worden geïdentificeerd als prognostische factoren. Samenvattend lijkt het erop dat behandelingsrespons en het klinisch beloop worden beïnvloed door veel verschillende biologische variabelen. Het zou getuigen van een tekort aan inzicht als we aan zouden nemen dat slechts één of twee biologische variabelen bijvoorbeeld het metastaseringsgedrag van een WDS of het klinisch beloop van een individuele patiënt zouden beïnvloeden. Nieuwe technieken zoals DNA-chip technologie kunnen behulpzaam zijn bij het vinden van een patroon van "risicofactoren" voor elke individuele tumor, zoals het aanwezig zijn van genmutaties of het tot expressie komen van oncogenen. Dit zou kunnen leiden tot klinisch relevante informatie zoals het voorspellen van behandelingsresultaten en metastaserings-potentie van de primaire tumor. Vanwege het feit dat WDS zeldzaam zijn, kan dit alleen worden bereikt binnen samenwerkingsverbanden.

Met het voortschrijden van het inzicht in de tumorbiologie is het een uitdaging om met behulp van moderne technieken nieuwe tumorbiologische variabelen te implementeren in de diagnostiek en in de keuze van de op de individuele patiënt toegesneden behandeling. Hierdoor zal het uiteindelijk mogelijk zijn de prognose van patiënten met een weke delen sarcoom verder te verbeteren.

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Dankwoord

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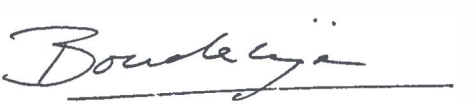
Iedereen, nogmaals: bedankt !



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Dag en Nacht

M.C. Escher (1898-1972) - februari 1938, houtsnede

" Ik probeer in mijn prenten ervan te getuigen dat we leven in een schone, geordende wereld, en niet in een nameloze chaos zoals het soms lijkt."
De prenten van Escher dienen " ... om een bepaalde gedachtengang duidelijk te maken. De ideeën die eraan ten grondslag liggen getuigen veelal van mijn verwondering over en mijn bewondering van wetmatigheden die de ruimte om ons heen bevat. Wie zich verwondert geeft zich rekenschap van een wonder. Door zintuigelijk open te staan voor raadsels die ons omringen en door mijn gewaarwordingen te overdenken en te analyseren, kom ik in de buurt van het domein van de wiskunde...".

M.C. Escher

Uit: "Leven en werk van M.C. Escher", Meulenhoff Amsterdam 1981

Tumor lijkt chaos, maar we gaan ervan uit dat deze zich volgens, nog niet geheel bekende, regels gedraagt. Zo probeert het onderzoek beschreven in dit proefschrift, deze regels te ontdekken door het bekijken van afgeleide beelden. Maar hoe interpreteren we wat we zintuigelijk waarnemen en wanneer mogen we iets "waarschijnlijk" noemen? De ene onderzochte figuur beïnvloedt namelijk de andere. Op welk moment wordt het duidelijk dat velden veranderen in vogels?
Pas als het de som der delen bekend is, wordt de geordende wereld, waar we naar op zoek zijn, duidelijk. Het ontdekken van de ordening. Daar houdt wetenschappelijk onderzoek zich mee bezig.

Vaak worden variabelen onderzocht die elkaars uitersten blijken te zijn. Het tussenliggende dynamische en grijze gebied is niet duidelijk te herkennen, maar wel af te leiden uit een analyse van die schijnbare chaos. Ondanks het voortschrijden van onze inzichten, blijven we nog steeds in het grijze midden. Pas wanneer het exacte verband tussen de bepaalde vormen duidelijk wordt en daardoor het contrast groter wordt, zal een compleet beeld ontstaan.

B.E.Ch. Plaat